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TOXICITY AND ENVIRONMENTAL IMPACT ASSESSMENT TESTS BASED ON THE ACTIVITY OF INDIGENOUS BACTERIA
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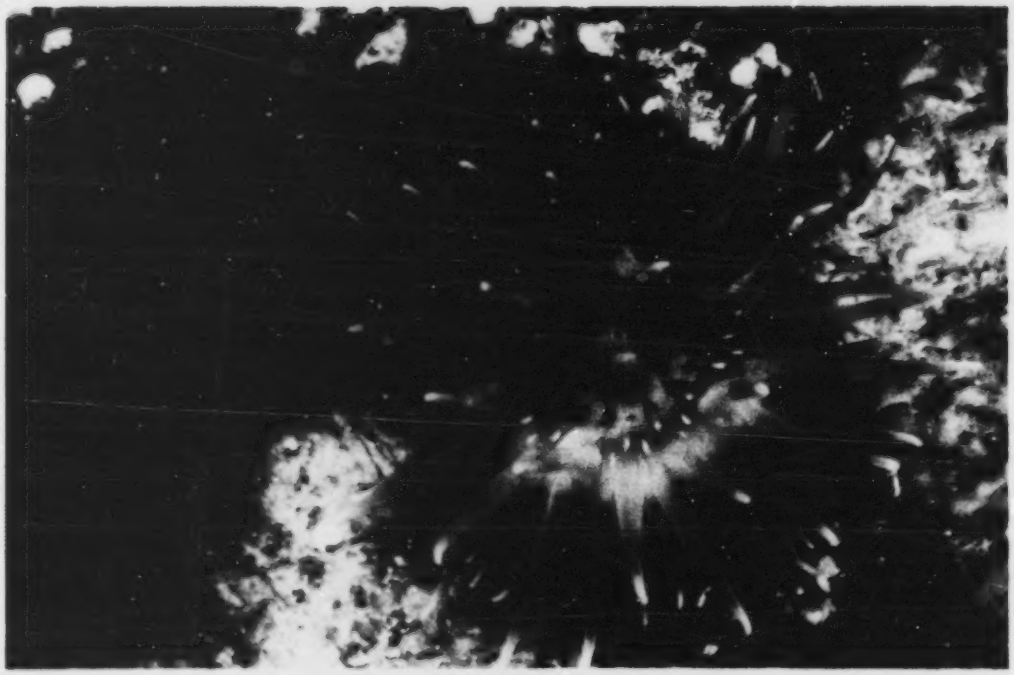
K. Lee, K.L. Tay, C.N. Ewing and E.M. Levy



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TOXICITY AND ENVIRONMENTAL IMPACT ASSESSMENT TESTS BASED ON THE ACTIVITY OF INDIGENOUS BACTERIA

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ABSTRACT

Lee, K., K.L. Tay, C.N. Ewing and E.M. Levy. 1990. Toxicity and environmental impact assessment tests based on the activity of indigenous bacteria. Ocean Dumping Report 4, Environment Canada: iv + 138 p.

Microorganisms in marine sediment mediate nutrient regeneration, trace metal transformation and organic matter degradation. A range of experimental techniques has been developed, using single indicator species, to evaluate the effects of contaminants on bacteria in sediment. There is, however, no evidence supporting the premise that the environmental impact of contaminants can be known from tests based on the toxicological response of single species. To provide a more sensitive, economical and rapid means of assessing the potential toxicity of substances, new procedures have been developed to measure the activity of indigenous assemblages of microorganisms with radiolabelled and fluorogenic substrates.

RÉSUMÉ

Lee, K., K.L. Tay, C.N. Ewing et E.M. Levy. 1990. "Tests d'évaluation de l'impact environnemental et de la toxicité basés sur l'activité des bactéries indigènes". Immersion de déchets en mer, rapport 4, Environnement Canada: IV + 138 p.

Les micro-organismes dans les sédiments marins servent comme médiateurs dans la régénération d'éléments nutritifs, la transformation des métaux traces et la dégradation des matières organiques. Une gamme de techniques expérimentales, chacune utilisant une espèce indicatrice particulière, a été mise au point pour évaluer les effets des contaminants sur les bactéries qui se trouvent dans les sédiments. Cependant, aucune preuve n'indique que l'impact environnemental des contaminants peut être démontré à partir de tests basés sur les réponses toxicologiques d'une seule espèce. Afin d'avoir un moyen plus sensible, économique et rapide d'évaluer la toxicité d'une substance, de nouvelles procédures ont été développées pour mesurer l'activité des assemblages de micro-organismes indigènes en utilisant des substrats fluorogéniques ou marqués radioactives.

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1.0 INTRODUCTION

Microbial processes are of vital importance in the aquatic environment. They are responsible for most chemical transformations in nature, including the degradation of organic compounds, regeneration of nutrients and transformation of trace metals (Bauer et al., 1981; Haines et al., 1982; Barnhart & Vestal, 1983; Lee et al., 1985; Degobbis et al., 1986). Until recently, however, the effects of contaminants on the microbiota have been virtually ignored in environmental impact studies. The ecological significance of microbial activity was not recognized and techniques to measure key metabolic processes were not available (Griffiths, 1983).

2.0 MICROBIAL RESPONSES MEASURED IN EXISTING TOXICITY AND IMPACT ASSESSMENT TESTS

The acute toxicity of compounds is traditionally defined in bioassay tests by the dosage which causes 50% mortality of test organisms (LD_{50}). However, contaminants in the environment are often present at concentrations, not acutely lethal, that cause chronic or sublethal effects, which cannot be quantified by the LD_{50} parameter (Liu & Thomson, 1983). Parameters such as respiration, enzyme inhibition and reproductive success are used to assess the sublethal effects of pollutants (Chapman & Long, 1983) defined by the effective concentration which causes a 50% reduction in the rate of the process studied (EC_{50}).

Bacteria are ideal test subjects for sublethal bioassays since they grow rapidly, are inexpensive, easy to maintain in culture and have enzymatic processes common to those of higher organisms (Bitton, 1983; Buikema et al., 1981; Liu, 1981; Dutka & Kwan, 1981).

2.1 Growth and Viability

Bacterial bioassays were originally based on observed changes in growth rates in response to toxicants. The growth of cultured bacteria was measured either turbidimetrically, by dilution and plating (Alsop et al., 1980; Burton & Lanza, 1985), or by direct counting (Polonenko et al., 1979). Typically, such common laboratory species as Pseudomonas fluorescens or Aeromonas hydrophila (Dutka et al., 1983), and mixed cultures isolated from activated sludge or sediment (Alsop et al., 1980) were used. In contrast to other assays of microbial activity, growth rate measurements require lengthy incubations during which a single concentration of toxicant is exposed to increasing biomass (Trevors et al., 1981). These long periods of incubation increase the risk of selecting resistant strains. This measures the effects of a toxicant on resistant strains, not the whole population (Barhart & Vestal, 1983).

The enumeration of viable colony forming units (CFU) on nutrient agar plates, inoculated with cultures exposed to different concentrations of toxicants, has also been used as a measure of lethal effects (Albright et al., 1972; Anderson & Abdelghani, 1980). However, CFU tests are reported to be more than an order of magnitude less sensitive to toxicants than other bacterial bioassays (Jonas et al., 1984). Furthermore, growth on nutrient agar does not provide reliable information on the effects of pollutants under natural conditions (Newell & Fallon, 1982; Griffiths et al., 1982b; Slabbert, 1986).

The "Bactericidal Disk Test" is another common, growth inhibition test. Small disks of filter paper, impregnated with concentrations of toxicant or defined droplets of toxicant, are placed directly on the surface of agar plates colonized with bacteria (Trevors and Basaraba, 1980; Thompson & Watling, 1985; Liu & Kwasniewska, 1981). Since growth inhibition is estimated from the size of the clear zone (attributed to growth inhibition) which develops around the toxicant droplet or the paper disk, the test is highly subjective.

In laboratory culture experiments, the activity of adenosine triphosphate (ATP) has been shown to be closely correlated with bacterial biomass (Jenkinson et al., 1979) and to be greatly inhibited by contaminants, such as mercury and acrolein (Kennicutt, 1980).

However, the analysis of ATP by the firefly luciferin-luciferase system (Burnison, 1975; Kennicutt, 1980) involves a lengthy extraction procedure that is relatively expensive and not very precise (Liu & Strachan, 1979). The procedure has not been widely adopted for toxicity testing.

The Spirillum volutans Motility Test for bacterial viability is based on the effective concentration causing cessation of flagellar motion in 90% of the indicator organism relative to a control treatment (EC_{90}) (Dutka et al., 1983). While, inexpensive and only slightly less sensitive than the Microtox test (refer to Section 2.4), with which it correlates well in terms of the ranking of toxic chemicals (Qureshi et al., 1982; Dutka et al., 1983), the procedure is subjective and labor intensive (Qureshi et al., 1982).

2.2 Respiration

Changes in rates of oxygen consumption, in both the water column (Thomas & O'Reilley, 1981) and sediments (Phoel et al., 1981) have been used to assess waste disposal impact. Unfortunately, few researchers have distinguished between chemical and biological uptake in their studies. This may be an important factor in sediments where researchers have reported incidents (e.g. when using bacterial inhibitors such as m-cresol) of chemical oxygen demand exceeding biological oxygen demand (Liu & Strachan, 1981). In addition, while microbial respiration tests have been reported to be more sensitive to toxicants than fish and amphipod assays (Busch, 1982) and bacterial-growth assays (Trevors & Basaraba, 1980), sensitivity appears to depend on the chemical tested. For example, Trevors (1982) reported that both O_2 consumption and CO_2 evolution were less sensitive measures of pentachlorophenol toxicity than the growth of Pseudomonas fluorescens.

Lengthy incubations must be avoided in respiration studies since bacteria can rapidly adapt to pollutants and in the case of organic contaminants, may use them as energy sources. For example, Griffiths et al. (1982a) reported that oxygen consumption in sediments was initially depressed upon exposure to crude oil but became increasingly stimulated after two days. Murray & Guthrie (1980) reported that the response of microbial respiration to

toxicants can vary between depression and stimulation within a chemical class. Given the above discrepancy and considering the view of Gillet et al. (1983) that the reproducibility is fair at best, bioassays based on respiration measurements are not recommended.

2.3 Genotoxicity

The Ames Mutagenicity Test (Ames et al., 1975), perhaps the most frequently used bacterial toxicity assay, is based on the reversion of specific strains of Salmonella typhimurium incapable of prototrophy, back to prototrophic forms, following exposure to mutagenic chemicals. It has been used with contaminated sediments (Dutka & Switzer-House, 1978; Samolloff et al., 1983; Van Coillie et al., 1985) and process effluents (Dutka et al., 1981). Unfortunately, highly toxic contaminants can decrease the total number of viable test organisms which is subsequently reflected in the number of revertant colonies, thereby decreasing apparent mutagenicity (Dutka et al., 1981). Together with this phenomenon questions about the relevance of mutagenicity to bacterial metabolism have led to decreasing popularity for this procedure in environmental assessment.

Recently, a new bacterial genotoxicity bioassay has been developed based on the expression of the SOS genes in Escherichia coli K-12, which regulates a group of enzymes induced by DNA damage (Quillardet et al., 1982). A commercial form of this bioassay, the SOS Chromotest (Orgenics Ltd.), has been used to evaluate the mutagenic potential of a wide range of chemicals (Quillardet et al., 1985). While results of the SOS Chromotest show excellent correlation with the Ames test, the procedure involves incubation periods up to 18 h (Mamber et al., 1986), and extraction and concentration of samples before genotoxic activity can be measured (Dutka et al., 1986). In an evaluation of the toxicity of Lake Ontario sediments, the SOS Chromotest did not correlate well with the results of the Microtox test, and failed to detect genotoxic activity in 13 of 15 sediment samples that had positive results in the Microtox assay (Dutka et al., 1986).

2.4 Bioluminescence

Changes in the bioluminescence of marine bacteria provide an indication of metabolic state since the light output by these organisms is produced using reduced flavin and oxygen in a branch of the electron transport chain (Chang et al., 1981). Microbial bioluminescence tests are usually conducted with Photobacterium phosphoreum (Bulich, 1979) or P. fischeri (Lebsack et al., 1981).

In 1977, Beckman Instruments Incorporated began to market the Microtox Assay System(R), a commercial system for conducting luminescence bioassays (Bulich, 1979). The system simultaneously measures the light output from a control and five toxicant treated samples, within 30 minutes (Bulich et al., 1981), at less than 5% of the cost for a standard (96 h) fish bioassay (Curtis et al., 1982). It has been applied to a wide range of toxicants including pure inorganic and organic compounds (Dutka & Kwan, 1981, 1982; Curtis et al., 1982; DeZwart & Slooff, 1983; Bulich et al., 1984; Cunningham et al., 1986; Cenci et al., 1987), complex process effluents (Chang et al., 1981; Coleman & Qureshi, 1985) and contaminated sediments (Schiewe et al., 1985; Williams et al., 1986). The ranking of toxic chemicals in Microtox assays is well correlated with LC_{50} bioassays conducted with algae (Dezwart & Slooff, 1983), daphnia (Bulich, 1982), fish (Greene et al., 1985; Chang et al., 1981; McFeters et al., 1983; Bulich et al., 1981) and rats (Chang et al., 1981). In direct comparison to other bacterial assays, the Microtox test has been reported to be more sensitive than growth rate, viability and enzyme assays (Cenci et al., 1987; Dutka & Kwan, 1981). Microtox assays with sediment pore water were used successfully by Giesy et al. (1988a, 1988b) to delineate contaminated and relatively clean areas of the Detroit River.

The optimal pH for the Microtox assay is 6.7. Adjustment of toxicant pH, before testing, is a major drawback in the assay since pH and redox conditions affect the chemical speciation of dissolved compounds (Stumm & Morgan, 1981) in aquatic systems. In terms of toxicity to the biota, it is the concentration of free metal ions, not the total concentration of metal present, that is of importance (Allen et al., 1980; Canterford & Canterford, 1980). While pH is known to affect the toxicity of pollutants (Dutka & Kwan, 1981,1982), with the exception of the study by Coleman & Qureshi (1985), Microtox bioassays have been

conducted under optimum pH conditions. There is considerable variation in the results of the Microtox assay between labs due to the complexity of the light output measuring device and variability between samples of lyophilized Photobacterium (Dutka and Kwan, 1981; Bulich, 1979). In pure compound assays, however, the Microtox test has generally been found to be both the most sensitive and replicable of the microbial assays (Greene et al., 1985; Elnabarawy et al., 1988). While it is highly recommended for routine monitoring of effluent quality or for primary screening of pollutants in a battery of tests (Lebsack et al., 1981; Bulich, 1982; DeZwart & Slooff, 1983; Slooff, 1985), it is unlikely that this bioluminescent assay will replace other bioassays, due to its inherent variability and dependence on neutral pH.

2.5 Nutrient Dynamics

Inorganic and organic nutrient cycles in aquatic environments are predominantly controlled by microbial activity (Morita, 1982; Griffiths, 1983). Effects of pollutants on primary nutrient cycle processes have received considerable attention following the demonstration of the adverse effects on these processes at concentrations much lower than those which elicited changes in population and community structure (Ausmus, 1984).

A wide range of ^{14}C- and ^{3}H- radiolabelled organic substrates (glucose, glutamate, aspartate, lactate, acetate, urea and thymidine) has been used successfully to measure heterotrophic activity in the aquatic environment (Litchfield et al., 1979; Novitsky and Kepkay, 1981; Fuhrman and Azam, 1982; Novitsky, 1983a, 1983b). There are two radiotracer techniques commonly used to measure the uptake of organic substrates, tracer and kinetic. Although the addition of any substrate represents a disturbance of the system being measured, in the tracer method, the substrate concentrations used are assumed to be so low that they do not significantly alter the ambient concentration (Wright & Burnison, 1979). This method enables one to calculate the actual uptake rate of the substrate if the in situ background concentration of substrate is known (Meyer-Reil, 1978, King & Klug, 1982). The kinetic approach involves the measurement of uptake rates over a range of substrate concentrations (Wright & Hobbie, 1965) and while more costly, the technique yields more

information since the maximal rate of uptake (V_{max}) and natural turnover time (T_t) can be determined using Michaelis-Menten kinetic analysis.

Toxic metals have been shown to depress ^{14}C-glucose incorporation (Albright et al., 1972; Mills & Colwell, 1977) and the water soluble fraction of crude oil has been shown to depress ^{14}C- glutamate uptake (Henson & Hayasaka, 1982). The typical response to toxicants is an increase in turnover time and a decrease in V_{max} (Albright et al., 1972). The use of ^{14}C-labelled substrates enables the researcher to determine how the substrate is utilized (e.g. tracing the incorporation of ^{14}C into fatty acids) and the fraction of substrate mineralized as $^{14}CO_2$ (Wright & Burnison, 1979). While respiration is often not measured in bioassays, it can account for up to 60% of the incorporated substrate (Hobbie & Crawford, 1969) and has been shown to be directly correlated with pollution stress. Griffiths et al. (1981) reported that after long-term exposure to crude oil, glucose and glutamate incorporation decreased while the proportion respired increased. Due to variations in biochemical utilization of different substrates, the effects of pollutants on the uptake of one substrate cannot be used to predict uptake rates for other substrates (Griffiths et al., 1977). Albright et al. (1972) reported a good correlation between glucose V_{max} and viable plate counts, however, with very toxic compounds, uptake was very low and kinetic parameters could not be calculated.

The effect of toxicants on biogeochemical cycling of nitrogen can be monitored during nitrification, nitrogen fixation and denitrification or both (Griffiths et al., 1982a). The process of nitrification (the oxidation of ammonia to nitrite and then to nitrate) measured by monitoring the concentrations of intermediates or products by colourimetric determinations of ammonia or nitrite (Williamson & Johnson, 1981); ammonia-specific probes (Neufeld et al., 1980); or ^{15}N radiotracers (Chatarpaul & Robinson, 1979) has been shown to be very sensitive to pollutants (Grundmanis & Murray, 1977; Mills & Colwell, 1977). Rates of nitrogen fixation and denitrification can be measured by gas chromatography (Knowles & Wishart, 1977; Capone et al., 1979; Yoshinari et al., 1977). Nitrogen fixation has been shown to be very sensitive to pollutants such as crude oil (Haines et al., 1981; Griffiths et al., 1982a).

Metabolic pathways in the sulfur cycle have not been studied extensively for use in environmental impact assessment. In a monitoring study of the effect of wood pulp mill effluent on sulfate reduction in sediments, Battersby & Brown (1982), by tracing the movement of $^{35}SO_4$ to $H^{35}S$, reported that sulfate reduction was an insensitive indicator of organic pollution.

Methanogenesis under anaerobic conditions has also been used to assess toxicity (Owen et al., 1979; Stuckey et al., 1980; Griffiths et al., 1982a). However, such assays involve lengthy incubation periods (30 d) and were judged by Sayler et al. (1982) to be of little value in toxicity assessment.

2.6 Activity of Specific Enzymes

The vital importance of enzymes at both cellular and ecosystem levels is universally recognized. In bacteria, for example, high molecular weight compounds must be enzymatically cleaved outside the cell wall before uptake can occur (Billen, 1984). Since the toxicity of most pollutants is attributed to enzyme inhibition (Riedel & Christensen, 1979; Buikema et al., 1980; Cairns, 1984; Vives-Rego et al., 1986), bioassays based on enzyme activity are generally more sensitive than population level (LD_{50}) tests (Liu, 1981), faster and more reliable than measurements of whole organism activity (Wieser & Zech, 1976).

The simplest enzyme assays are based on the interaction of pure enzyme and toxicant in vitro. Typically, enzyme and toxicant are mixed and the activity of the enzyme is measured colourimetrically as substrate disappears or products accumulate. The responses of a number of enzymes, including carbonic anhydrase (Christensen & Tucker, 1976), adenosine triphosphatase (Riedel & Christensen, 1979), glucose-6-phosphate dehydrogenase (Rutherford et al., 1979) and cholinesterase (Tabata et al., 1984), have been measured by this fashion. However, in some cases, in vitro activities of such enzymes as glucose-6-phosphate dehydrogenase have been shown to correlate poorly with fish LD_{50} values (Schultz & Harman, 1980).

There is little evidence to suggest that the activity of an enzyme in vitro has any relevance to its activity in a living system under natural conditions. While direct measurement of enzyme activity in sediments may give a better indication of their ability to degrade organic pollutants, the measurement of enzyme induction rates in organisms, stressed by pollution, may be the most ecologically relevant test. Tabata et al. (1986) measured enzyme activity induction in rat liver after injection of extracts from polluted river sediments. Benzene hydroxylase induction in fish has successfully been used as a sublethal test for crude oil contamination (Payne, 1976). The activity of mixed function oxidase (MFO) enzymes in fish has been suggested as a means of assessing pollutant impacts (Addison, 1984). While these techniques are ecologically more relevant than in vitro tests, they entail use of higher organisms and are labor intensive.

A number of techniques has been reported for the measurement of specific enzyme activities in cultures and natural populations of bacteria. These tests are primarily colourimetric in principle and have included dehydrogenases (Nachlas et al., 1960), phosphatases (Morita & Howe, 1957), amylase, cellulase, urease (Duddridge & Wainwright, 1982), proteases (Burton & Lanza, 1985) glucosidases and acetylglucosidase (Kanazawa & Filip, 1986). The inducible dehydrogenase enzymes which are responsible for feeding electrons into the electron transport system have been chosen for the study of toxicant impacts since they are involved in vital cellular anabolic and catabolic processes (Wieser & Zech, 1976; Liu & Thomson, 1983). Dehydrogenase activity is usually measured by the reduction of one of a number of tetrazolium salts to form the dye formazan (Burton & Lanza, 1985).

The most common assay, based on the use of triphenyltetrazolium chloride (TTC), requires long incubations (24h) and extraction of the formazan complex which is insoluble in water. The measurement of dehydrogenase activity with TTC has been shown to yield EC_{50} values similar to oxygen consumption (Dutka et al., 1983), and to be no more sensitive than viable plate counts (Reichardt, 1979b). As a result of the redox conditions required for the TTC-formazan reaction, the assay is unsuitable for use in anaerobic conditions (Ryssov-Nielsen, 1975). Another substrate, 2-(p-iodophenyl)-3- (p-nitrophenyl)-5-phenyl tetrazolium chloride (INT), was said to be a better indicator than TTC since it competes

well with oxygen for electrons, requires only a 2 h incubation, and has a redox balance that makes it appropriate for anaerobic conditions (Olanczuk-Neyman & Vosjan, 1977; Reichardt, 1979a).

More recently, the reduction of resazurin has been used to quantify dehydrogenase activity. Resazurin reduction requires only 90 min of incubation and is an order of magnitude more sensitive to toxicants than viable plate counts (Liu, 1981). There are conflicting reports about the degree to which dehydrogenase activity correlates with other bacterial assays. While it has been shown to correlate well with biomass and ATP concentrations in sediments by Liu & Strachan (1979), Sparling (1981) reported poor correlation with respiration, biomass, ATP concentrations and amylase activity in soils. These discrepancies may be due to use of different substrates (resazurin and TTC) by the individual laboratories. Furthermore, the relevance of pollutant effects on the electron transport system is not known (Bell & Ahlgren, 1987).

Enzyme assays have recently been developed which use enzyme specific substrates to avoid interference caused by natural, chemical reduction processes. For example, assays based on the release of p-nitrophenol from p-nitrophenyl phosphate by acid and alkaline phosphatases (Morita & Howe, 1957; Tabatabai & Bremner, 1969) have been shown to be sensitive to organic pollutants (Verstraete et al., 1976; Griffiths et al., 1982b). Similar colourimetric assays, based on nitrophenyl release from appropriate substrates, have been developed for B-glucosidase (Meyer-Reil, 1981), B-galatosidase (Cenci et al., 1985) and B-acetylglucosidase (Kanazawa & Filip, 1986). Glucosidase activity has been shown to be well correlated with ATP concentrations and respiration, and to be more sensitive than other bacterial activity measures, such as acetate incorporation (Barnhart and Vestal, 1983).

While there is evidence of good correlation between enzyme tests and bioassays with higher organisms (Buikema et al., 1980), great differences in the sensitivity between different enzyme assays have been reported. For example, Armant et al. (1980) reported that glucose-6-phosphate dehydrogenase was much less sensitive to toxicant additions than either phosphatase or amylase. Dehydrogenases and growth rate have been shown to

sensitive to chlorophenols while B-galactosidase was relatively insensitive (Cenci et al., 1987). Griffiths et al. (1982b) reported a depression in phosphatase, cellulase and chitobiase activities and stimulation of laminarinase, amylase and alginase activities in marine sediments in response to crude oil. Inconsistency in sensitivity patterns is undoubtedly due to the physicochemical variation between enzymes and differences in the modes of action by toxicants.

The measurement of enzyme activity in sediment samples is complicated by numerous factors including pH, temperature, concentration and refinement techniques (Burton & Lanza, 1985). When sediment is present in an assay vessel, problems arise due to binding and inactivation of enzymes by sediment particles (Buikema et al., 1980). Lengthy incubations in some assays have led researchers to use such bacteriostats as toluene to prevent bacterial growth and synthesis of new enzymes during the assay (Verstraete et al., 1976; Duddridge & Wainwright, 1982). However, where inducible enzymes (enzymes synthesized or activated only in the presence of suitable substrate), such as dehydrogenases, are involved (Liu & Strachan, 1979), active cell metabolism must be maintained.

Only potential rates of enzyme activity can be measured using colourimetric enzyme assays techniques requiring incubation with high concentrations of suitable substrates since natural enzyme activity levels are below the limits of detection. Most enzyme assays are buffered at the optimal pH of the enzyme system in question and thus, the results cannot be extrapolated to nature with any certainty. Conversely, while unbuffered enzyme assays yield more ecologically relevant results they are susceptible to pH shifts resulting from product accumulation (Duddridge & Wainwright, 1982).

3.0 APPLICABILITY OF SINGLE SPECIES TESTS

Some researchers have argued that the use of pure cultures of bacteria eliminates "anomalous results" due to the differences in sensitivity by individual organisms in mixed culture (Thompson & Watling, 1985). However, there is no evidence to support the premise that the environmental impact of a toxicant can be predicted with a single species

test (Cairns, 1984). It is important that future efforts at predicting pollutant impact be directed towards development of assays based on higher levels of organization and diversity than a single species in isolation (Ausmus, 1984). Tests involving entire communities and ecosystems must be designed to reflect natural diversity (Neuhold, 1986; Bayne, 1981).

The standard organisms used in single species tests, such as rainbow trout (Salmo gairdneri) or bacteria (eg. Photobacterium sp.), are often not representative of the indigenous biota (Chapman & Long, 1983). Standard test species, easily maintained under laboratory conditions, are usually strains with great resistance to stress. Patterns of sensitivity differ both between and within species of bacteria to various classes of toxicants (Bringmann & Kuhn, 1980; Qureshi et al., 1982). For example, Kenaga (1978) reported that toxicity values for single species tests varied between 0.2 and 2.5 orders of magnitude (with 95% of the values falling within 1.4 orders of magnitude) and suggested that differences between tests should be greater than one order of magnitude before being judged significant. As a result of these discrepancies, Dutka & Kwan (1981) concluded that if single species tests are to be used, a battery of tests, including at least 2-3 genera and 2-4 species should be used.

While mixed cultures of bacteria isolated from water (Murray & Guthrie, 1980; Vives-Rego et al., 1986), wastewater (Alsop et al., 1980), and soil or sediment (Mowat, 1976; Anderson & Abdelghani, 1980; Liu, 1981) have been used in bioassays, these cultures were typically maintained in nutrient media. The results obtained cannot be used to accurately predict toxicant impacts under natural conditons (Jonas et al., 1984).

In order to obtain accurate predictions of toxicant effects, it is essential to use samples collected from the area of concern (Barnhart & Vestal, 1983). The best predictions would be obtained from in situ studies. This is not always practical, however, due to logistics. The alternative recommended is to conduct laboratory experiments with natural samples maintained under ambient conditions.

4.0 SEDIMENT BIOASSAYS

Marine sediments are primary sites of nutrient cycling, organic matter degradation, trace metal transformation and oxygen depletion in the aquatic ecosystem (Liu & Strachan, 1981; Griffiths et al., 1982b). While sediments represent the ultimate sink for aquatic nutrients and contaminants, the ecological significance of toxicant inhibition on the microbial activity of sediments is not clear (Burton & Lanza, 1985).

Marine sediments are complex mixtures of different-sized particles, both inorganic and organic, containing microgradients of nutrients and organic carbon. The upper surface of sediments is usually characterized by a thin layer of flocculent organic matter that consists of fecal pellets, dead phytoplankton and other organisms, including bacteria (Harrison et al., 1971). Heterotrophic activity at the sediment-water interface is twice that of deeper sediment layers and two to three orders of magnitude higher than in overlying waters (Novitsky, 1983a). In addition, peaks in bacterial numbers and biomass have been found throughout the sediment column around pockets of detritus (Meyer-Reil, 1984). Hydrolases and oxidoreductases are the major enzyme systems active in aquatic sediments (Butron and Lanza, 1985). The impact of contaminants on these enzyme activities has received the greatest attention. Many of these enzymes can become bound to humic acids and form stable complexes that remain active in sediments for extended periods (Meyer-Reil, 1981).

In sandy sediments, 80 to 95% of the bacterial population is attached to the sediment particles, while in muddy sediments, as much as 50% of the total population may be free in the interstitial water (Wieser & Zech, 1976; Meyer-Reil, 1984). Of this total population, only a fraction is typically active in sediment processes (Kirchman, et al., 1985; Novitsky, 1983a). In comparison to overlying waters, bacterial activity in sediments may be lower on a per cell basis, but overall productivity values may be higher due to the number of organisms present (Newell & Fallon, 1982).

In standard bioassay procedures, the physical nature of sediment often creates interference in colourimetric analyses and thus, filtration or centrifugation procedures are often

required. Furthermore, most buffers used in enzyme assays extract colored compounds from the sediments, mostly humic and fulvic acids, which can cause interference in analyses (Tabatabai & Bremner, 1969; Duddridge & Wainwright, 1982).

Binding of enzymes and labelled substrates to sediment particles has been shown to result in decreased activity and deviation from Michaelis-Menten kinetics (Irving & Cosgrove, 1976; Armant et al., 1980; Kanazawa & Filip, 1986; Findlay et al., 1984). While increasing solubility, processes such as chelation of metals to organic compounds tend to decrease toxicity (Allen et al., 1980; Canterford & Canterford, 1980). In order to alleviate these problems, researchers have attempted to extract the contaminants from the sediment for use in toxicity assays. Unfortunately, extraction procedures have not been developed, not that they are likely to be, to the point where quantitative extraction of all contaminants is achieved.

The most commonly used extraction procedure is the elutriation technique. Sediment elutriation was developed by the U.S. Army Corps of Engineers and the U.S. Environmental Protection Agency as a method to assess the release of contaminants during dredging and disposal activities. Elutriation involves mixing and aeration of a sediment water mixture; after settling, the supernatant passes through a 0.45um pore size filter (Mudroch & Davies, 1985). Unfortunately, the high redox conditions prevailing during elutriate preparation tend to prevent release of large proportions of the metals present in a dissolved state.

Elutriates have generally not been found to be good predictors of sediment toxicity since much better correlation exists between toxicity and sediment chemical composition than between toxicity and elutriate composition (Laskowski-Hoke & Prater, 1981b). While water extracts, such as elutriates, may extract some metals and polar organic compounds, they cannot be used to assess the impact of neutral, non-ionic compounds, such as aromatic or chlorinated hydrocarbons (Williams et al., 1986). Solvents such as dimethyl sulfoxide (DMSO) and hexane-dichloromethane have been used to extract the toxic fractions from sediments (Samolloff et al., 1983; Tabata et al., 1984; Schiewe et al., 1985), however, their inability to extract different classes of contaminants simultaneously, remains a serious

drawback. Furthermore, physical exposure to sediments appears to be important in bioassays (Laskowksi-Hoke & Prater, 1981a; Powers et al., 1982), since quantitative and qualitative differences in responses to sediments and elutriates have been reported (Peddicord & McFarland, 1976; Ewing & Nalewajko, 1987).

While the collection of sediment samples by coring is less disruptive than other methods, all sampling procedures will affect sediment fine structure and associated microgradients (Bell & Ahlgren, 1987). Disturbance of sediments by sampling procedures has been demonstrated to cause changes in measured metabolic rates such as heterotrophy and phospholipid synthesis (Meyer-Reil, 1984; Moriarty et al., 1985). However, some measures of bacterial metabolism, such as thymidine incorporation, do not respond quickly to disturbance (Bell & Ahlgren, 1987). Novitsky & Kepkay (1981) reported increased heterotrophy at the aerobic and anaerobic horizon in sediment cores. Furthermore, aerobic sediments have been shown to recover from pollutant addition much faster than anaerobic communities (Bauer & Capone, 1985). For a relatively valid representation of the environment, it is suggested that experiments be conducted with intact sediment cores maintained under natural conditions during the assay procedures.

Much of the experimental research on microbial activity in sediments uses sediments suspended in water (slurries), in ratios of 1:1 to 1:1000 (Griffiths et al., 1977; Novitsky, 1983a; Boon, 1986). This procedure simplifies the addition of labelled substrates during the assays and ensures their even distribution throughout the sediment. However, due to the physical disruption of sediments, the measured rates of microbial activity represent metabolic responses to different nutrient and substrate concentrations by microorganisms homogeneously distributed in the experimental system (Harrison et al., 1971). Consequently, the measurement of microbial activity in intact sediment cores (Meyer-Reil, 1978, 1986; Ansbaek & Blackburn 1980; Novitsky, 1983b), a more ecologically sound approach, has recently been adopted. Metabolic activity has been shown to be approximately one order of magnitude lower in intact cores than in slurries (Novitsky, 1983; Meyer-Reil, 1984). In an attempt to minimize the disruption of sediments in cores while adding substrates homogeneously, Meyer-Riel (1978) and Novitsky (1983b) used low vacuum to draw the substrate into cores. However, this procedure replaces the interstitial

water in the sediment cores and tends to disrupt microgradients. Recent studies have suggested micro injection (5-10 ul) of substrate into intact cores at short (0.5-2 cm) intervals (*Ansbaek & Blackburn, 1980; Meyer-Reil, 1986*).

In numerous bioassay tests, the significance of chemical reduction of dye complexes or oxygen consumption by chemical reactions has not been adequately addressed. Natural chemical reactions in sediments can completely invalidate assays based on simple reduction of reactants, if corrections to differentiate them from biological (enzymatic) reduction are not performed. Meyer-Reil (1981) attempted to differentiate between chemical and biological reduction by running parallel series; one of which was poisoned with a bacteriostat such as m-cresol or formaldehyde. Biological activity was defined as total activity minus chemical reduction. However, Reichardt (1979a) has shown that formaldehyde and phenolics, such as cresol, only inhibit about 50% of the activity of succinic dehydrogenase and that such bactericides could interfere with chemical reduction of the dye complex, causing an overestimation of biotic activity. These findings highlight the importance of employing enzyme specific substrates that are not easily broken down by simple chemical processes in sediments.

Assays of sediment microbial activity are often plagued by the question of what constitutes an appropriate control. Meyer-Reil (1986) reported both high and variable values for enzyme activity in controls poisoned with formaldehyde. While autoclaving kills bacteria in sediment and arrests enzymatic activity (Ladd & Butler, 1972; Somville & Billen, 1983; Billen, 1984), recent experiments have demonstrated that even short heating periods (85°C, 5 min.) cause significant changes in the physical and chemical properties of the sediment (Ewing, 1987). Furthermore, Dutka & Switzer-House (1978) have attributed the toxicity in sediment to heat labile compounds.

5.0 RESEARCH CONDUCTED UNDER THE OCEAN DUMPING RESEARCH FUND

In response to the outlined weaknesses in existing microbial test procedures, the goal of the present project, supported by the Ocean Dumping Research Fund, was to develop and evaluate a series of microbial activity assays for use in toxicity and environmental impact assessment studies. The criteria for these studies are as follows:

1) to be based on the activity of primary metabolic processes found in most organisms;
2) measure the activity of indigenous biota under natural conditions;
3) be performed rapidly, inexpensively and simply;
4) require short incubation periods with no adjustment of pH in the test system;
5) avoid interference from natural chemical reactions by being highly enzyme specific;
6) produce results that are reliable and reproducable;
7) sensitivity to a wide range of contaminants and correlation with existing bioassays;
8) feasible for use under field conditions;
9) be implemented into existing toxicity/impact assessment programs.

5.1 Enzyme Specific Bioassays

Many of the problems inherent in colourimetric enzyme assays have recently been avoided with the use of substrates yielding highly fluorescent products. Fluorogenic assays are generally faster, simpler and cheaper to perform than conventional colourimetric assays. The first fluorogenic enzyme assay was described by Perry (1972) using 3-o-methylfluoro-scein phosphate to assess phosphatase activity. More recently, fluorescent 4-methylumbelliferone (MUF) substrates have become popular and used to measure not only phosphatase (Petterson & Jansson, 1978), but glucosidase (Hoppe, 1983; Slifkin & Gil, 1983; Somville, 1984), protease (Somville & Billen, 1983) and chitinase (O'Brien & Colwell,

1987). There is no need to account for chemical breakdown of substrates during assays because MUF substrates are highly enzyme specific (Hoppe, 1983).

Compared to chromogenic substrates, the high sensitivity of fluorogenic substrates allows much shorter incubation times and measurement of low activity without the use of high enzyme and substrate concentrations (Petterson & Jansson, 1978; Somville & Billen, 1983). Also, better sensitivity is achieved (Somville, 1984) and enzyme activities can be measured at their ambient, not potential rates (Meyer-Reil, 1986), because fluorogenic substrates are generally used at concentrations three orders of magnitude lower than chromogenic substrates (Perry, 1972).

In addition to MUF substrates, naphthylamine substrates have been used to measure proteolytic activity (Vives-Rego et al., 1985; 1986). Results correlate well with thymidine incorporation (a measure of bacterial growth). Fluorogenic measurements of glucosidase activity have been shown to correlate well with the incorporation of organic substrates including glucose, thymidine and amino acids (Somville and Billen, 1983; Somville, 1984). To date, fluorogenic substrates have only been used to assess enzyme activity in specific environments; no attempt has been made to incorporate them into bacterial bioassays.

Experiments conducted during the development of enzyme bioassays with MUF-conjugated substrates are briefly outlined below. Final test procedures for the assays are described in detail in Appendices A to C.

5.2 Preliminary Experiments

5.2.1 Michealis-Menten Kinetics

Objective: To determine if B-glucosidase activity in marine sediments follows Michaelis-Menten kinetics and to determine the optimal concentration of MUF-glucoside substrate for use in the enzyme bioassays.

Methods and Results: Slurries of sandy sediment (0.2 g sediment in 3.0 ml synthetic ocean water), with a range of MUF-glucoside concentrations (0.5 to 100uM), were incubated at ambient temperature for 1 hour, after which the activity was terminated by freezing. Activity was deduced from the fluorescence of liberated MUF.

Activity appeared to follow Michaelis-Menten kinetics (Fig. 1). Due to enzyme induction, a linear increase in enzyme activity with increasing substrate concentration was evident at higher concentrations and a true V_{max}, the maximum velocity of enzyme activity, was not observed. Lineweaver-Burke linear transformation of the data yielded a theoretical V_{max} of 8.3 uM/g/h and a half saturation constant (K_m) of 11.0 uM. There was no measurable activity in autoclaved controls. Calculation of the MUF standard curve showed that the fluorescence of MUF was linear with concentrations up to 1.0 uM.

Conclusions: It is important to use substrate concentrations as low as possible to avoid significant increases in the ambient concentrations of naturally occurring substrates. Thus, major disruption of the chemical conditions in the sediment is avoided and the ambient, not the potential, enzyme activity is measured (Meyer-Reil, 1986). It was concluded that 5 uM MUF-glucoside should be used in future experiments since this amount produced concentrations of methylumbelliferone which could be measured directly without dilution.

5.2.2 Linearity of Enzyme Activity

Objective: To demonstrate that the activity of the enzyme systems is linear over the experimental incubation period for endpoint type experiments. To determine if any residual enzyme activity is present in the sediment before substrate addition.

Methods and Results: Triplicate sets of sediment slurries (0.2 g sediment in 3.0 ml synthetic ocean water) were set up with the following experimental treatment immediately prior to substrate addition: (1) formalin fixed (15%); (2) autoclaved (20 min at 121°C); and (3) untreated control. MUF-glucoside was added (5uM) to all of the samples and three

tubes from each set were terminated by freezing at 30 min intervals.

Exoenzyme activity in the untreated sediment was linear at a rate of 1.23uM/g/h over a two h experimental period (Fig. 2). Enzyme activity in the formalin-fixed and autoclaved sediment was found to be very low (<0.09uM/g/h), in comparison.

Conclusions: Exoenzyme activity (B-glucosidase) in sediment slurries was linear ($P<0.05$) for at least two h. Based on the data obtained, a conservative incubation time of 60 min was adopted as a standard for the experimental bioassays. However, the incubation time could be extended to 120 min in situations displaying low activity (e.g. low temperature environments). The low enzyme activity detected in the formalin-fixed and autoclaved samples is attributed to residual enzyme concentrations present in the sediment before substrate addition. The small difference in enzyme activity between the formalin-fixed and heat killed sediment slurries is attributed to the denaturing of proteins by the autoclaving process. Formalin would kill the bacteria and prevent enzyme production but, presumably, not interfere with enzyme already present in the sediment. Low activity in the fixed samples also suggests that there is no chemical interference with the MUF-glucoside and MUF reaction in the experimental procedure.

5.2.3 Background Enzyme Activity

Objective: To determine the proportion of total enzyme (phosphatase) activity attributed to residual enzyme concentrations present in natural sediment and that induced in response to the addition of MUF-substrates. To assess the effect of a toxicant (Cu) on the concentration of residual and induced enzyme activity as measured using MUF-substrates.

Methods and Results: Sandy sediment was suspended in 15 parts of synthetic sea water (3 ml total volume) in duplicate sets. One set was filtered immediately on 0.22um filters and the filtrate caught in test tubes. In addition to untreated control samples of both whole sediment slurries and filtrate, formalin (3%) and copper (50 mg/L) were added to experimental samples. MUF- phosphate (10uM) was added and the samples were

incubated for 60 min at ambient temperature.

In the untreated control samples, induced activity (total background) accounted for 82% of the total observed activity (Fig. 3). In comparison, the addition of formalin caused an 87% decrease in induced activity and a 70% decrease in the background activity (filtrate) of the treated samples, resulting in an 84% depression of total activity. Copper (50 mg/L) addition resulted in an 82% depression of total activity, and an 83% depression of induced activity relative to the untreated control. The addition of copper and formalin decreased the proportion of total activity attributable to enzyme induction from 82% to 74% and 66%, respectively.

Conclusions: Analysis of exoenzyme activity (phosphatase) using an MUF-substrate indicated that residual enzyme activity only accounts for a small proportion of the total activity. In support of our assumption that these fluorogenic substrates can be used for bioassay tests, the induction of phosphatase exoenzyme activity appeared very sensitive to the addition of copper. While formalin suppressed residual enzyme activity, the filtration experiment showed that the addition of formalin did not completely suppress the induction of enzyme activity. These effects could be due to the activity of organisms which passed through the 0.22um filters (Li, 1987) or to artifacts caused by filtration.

5.2.4 Effect of Pre-incubation Time

Objective: To determine the effects of toxicant pre-incubation time on observed toxicity.

Methods and Results: Phosphatase activity was measured in whole slurries and 0.22um filtrates of sandy sediments, following the addition of a toxicant (Cd) at time intervals ranging from 0.5 to 4 h.

A rapid decline in enzyme activity (72%) was observed within the first 30 min (Fig. 4), after which enzyme activity decreased very slowly.

Conclusions: It is necessary to incubate the test organism with known doses of toxicant for a defined period prior to activity measurement. To avoid experimental artifacts, incubation times should be kept as short as possible. A 30 min pre-incubation period is widely used in other bacterial bioassays (Albright et al., 1972; Barnhart & Vestal, 1983) and since the greatest inhibition was observed within 30 min, this pre-incubation period was selected for use in the bioassay test.

5.3 Toxicity Assessment in Sediment Slurries

Objective: To assess the use of exoenzyme activity (based on the application of MUF substrates) as bioassay tools with a number of known toxicants.

Methods and Results: The responses of sediment bacteria to the six metals (Cu,Hg,Cd,Pb,Zn,Ni) classes under the Canadian Environmental Protection Act (CEPA) as prohibited or restricted substances were assessed in slurries of sandy sediment. Details of the method used are given in Appendix A. Confidence intervals (95%) for the EC_{50} estimates were calculated using the method of Sokal and Rohlf (1969).

Glucosidase (Figs. 5, 6; Table 1) showed a toxicity order of Hg > Zn > Cu > Cd > Pb = Ni while the 95% confidence intervals and the high correlation coefficients (> 0.957) indicated low variability between replicate slurries and a high degree of accuracy in the EC_{50} estimates. EC_{50} values obtained for phosphatase (Figs. 7, 8; Table 1) were generally higher than that for glucosidase and had a different toxicity order (Cu > Zn > Ni > Pb > Cd > Hg). The effect of mercury on phosphatase activity was very weak and the EC_{50} was well outside the range of concentrations used. The toxicity order of protease (Cu > Pb > Zn > Ni > Cd > Hg) was quite similar to that shown by phosphatase (Fig. 9, 10; Table 1), although the variance between replicates was much lower.

The effects of the toxic metals on the activity of three exoenzymes (glucosidase, phosphatase and protease) were assessed in fine sediment (mud), using the method outlined in Appendix A. Glucosidase exhibited a toxicity order

(Hg=Cu>Zn>Cd=Ni>Pb) similar to that shown in sandy sediment, but the EC_{50} values were higher in the muddy sediment for all metals except copper (Figs. 11, 12; Table 3). In the phosphatase experiment, a different sequence in toxicity order and slightly lower EC_{50} values were observed in the muddy sediment (Figs. 13, 14; Table 3). Protease showed a toxicity order (Cu>Zn>Pb>Hg>Ni>Cd) (Figs. 15, 16; Table 3) very similar to that for phosphatase, while the EC_{50} values were lower. The correlation coefficients of the linear regressions were similar to those obtained with sandy sediment, indicating a similar degree of variability between replicates.

Conclusions: The activity of three exoenzymes in natural sediments was used to assess the toxicity of a series of known toxic metals. Glucosidase and protease were the most sensitive to the toxicants tested (Cd,Cu,Hg,Ni,Pb,Zn) and showed the lowest variability between replicate slurries. They provide the most accurate estimates of EC_{50} values. The slight differences in toxicity orders and EC_{50} values between the three enzymes are most likely due to differences in the various metabolic pathways involved. The values obtained for glucosidase were very similar to those obtained by Barnhart and Vestal (1983) in river sediments using colourimetric substrates (Table 2).

The use of MUF-substrates to assess exoenzyme activity in muddy sediments yielded results similar to those obtained with sandy sediment collected simultaneously from the same region (Long Cove, Nova Scotia). Higher EC_{50} values in the slurries of muddy sediment were probably due to higher concentrations of organic carbon in the sediment binding with free metal ions. While this organic chelation tends to increase the solubility of metals in aqueous solutions, it also reduces their toxicity since it lowers the effective concentration of the toxic free metal ions (Allen et al., 1980).

The results clearly indicate that the induction of exoenzyme production in sediment bacteria is very sensitive to pollutants and can be used in bioassays and environmental impact assessments. Tests with slurries of sediments can be completed in as little as 2.5 h. The great similarity of the toxicity orders between the bioassays with the three enzyme substrates indicates that any of the three could be used to test the potential impact of a toxicant. However, the use of all three substrates, as part of a battery approach, would give

a more accurate estimate of toxicity.

5.4 Toxicity Assessment in Sediment Cores

5.4.1 Development of Techniques

Objective: To ascertain if, once perfected, the techniques of utilizing MUF-substrates as bioassay tools could be applied to intact sediment cores. To determine the extent to which an injected toxicant and enzyme substrate diffuse through a core during the incubation period, and whether the toxicant and substrate were evenly distributed in the sample core removed for analysis (see Appendix B).

Methods and Results: Zinc and MUF-leucine were injected directly into sediment cores at regular intervals throughout their depth. After incubation the cores were frozen and the center of each 1 cm horizon was removed to measure enzyme activity, as in sediment slurries. Details of the method used are given in Appendix B. Dose response curves and EC_{50} values were calculated for each sediment horizon. Enzyme activity was highest in the top 2 cm of the sediment core, gradually decreasing with depth (Fig. 29). The addition of zinc resulted in a linear decrease in exoenzyme activity in all sediment horizons. The EC_{50} values (Figs. 30, 31; Table 5) were similar throughout the core at approximately an order of magnitude lower than the values previously obtained in sediment slurries (Tables 1, 4). Variability between replicate cores, highest in the controls, was much lower if they were allowed to settle prior to use for at least 8 h after collection.

To assess horizontal transport through the core, 10 uL aliquots of a radiolabelled organic (^{14}C-glutamic acid; 0.22 uCi/mL) were injected into the center of triplicate cores at 5 mm intervals. After 30 min, the cores were frozen and sectioned into 1 cm horizons. Concentric plugs of 3, 8 and 12.5 mm diameter were cut from each horizon and placed in scintillation vials. The activity was measured as described in Appendix B. The labelled solution diffused through the core and was evenly distributed in the core, up to 4 mm from

the center (Table 6). However, only a small proportion (approximately 15%) had diffused past the 4 mm point.

To assess the extent of vertical diffusion, isotope (^{14}C-glutamic acid) was injected at 10 mm intervals and incubated for 30 min. After freezing, cores were sectioned into 2 mm horizons; each horizon was placed into a scintillation vial for measurement as above. Isotope diffused well through the cores and approximately 45% of the activity at the injection points was present midway between the injection ports (Fig. 32). Since the injection interval was twice that normally used (5 mm), the substrate concentration midway between injection points would be approximately 75% of that at the injection points, if injected at the normal intervals.

Conclusions: The activity of exoenzymes can be measured successfully in intact sediment cores. In the case of protease activity analysis, activity was much lower compared to sediment slurries. The EC_{50} was also much lower. Greater sensitivity of bacteria in intact cores highlights the importance of employing bioassay protocols that accurately model natural conditions.

The diffusion of substrate through the cores of sandy sediment was sufficient to ensure that a 5 mm plug in the center of each horizon would have evenly distributed substrate and toxicant concentrations. Thus, it was concluded that the core injection technique, which simulates the distribution of toxicants throughout surficial sediments by tidal flushing and bioturbation, was a valid method for toxicant and substrate introduction. If a larger diameter plug was desired (in cases of low activity), the sediment should be tested as above, to ensure adequate diffusion of the toxicant and substrate.

5.4.2 Metal Toxicity in Cores

Objective: To measure the responses of sediment bacteria in intact sediment cores to different toxicants.

Methods and Results: The core injection technique (Appendix B) was used to test the response of glucosidase activity to the six metals. The addition of copper to intact cores resulted in linear decreases in enzyme activity in all sediment horizons (Fig. 33), however, the EC_{50} was above the highest concentration used (Figs. 34, 35; Table 7). Mercury addition resulted in great depression of enzyme activity throughout the sediment cores (Fig. 36). The EC_{50} values were very low and similar in each sediment horizon (Figs. 37, 38; Table 7). The presence of nickel inhibited glucosidase activity to a great extent (Fig. 39) and the EC_{50} values calculated were uniform and very low, compared to other values obtained for nickel in sediment slurries (Figs. 40, 41: Table 7).

Both cadmium and lead caused much less inhibition than the other metals and neither reached the calculated EC_{50} within the range of toxicant concentration used (Figs. 42-47; Table 7). Since the calculated EC_{50} values greatly exceed the highest concentrations used, these values can only be used as estimates and cannot be considered absolute. Zinc inhibited enzyme activity throughout the sediment cores (Fig. 48) and the EC_{50} values calculated (Figs. 49, 50; Table 7) were slightly lower than those obtained with sediment slurries (see Tables 1 and 4). The toxicity order was the same as that obtained in experiments with sediment slurries (Hg > Cu > Ni > Zn > Pb = Cd).

Conclusions: The toxicity of compounds can easily be measured in sediment cores. The EC_{50} values obtained were generally lower than those obtained with sediment slurries, suggesting a greater sensitivity of bacteria in intact cores than in slurries. However, since sediments used in the slurry and core experiments were from different sources, direct comparisons cannot be made. The sediment used in the core experiments was from the intertidal zone of a very active sandy beach. This undoubtedly explains the great homogeneity in the EC_{50} values throughout the depth of the cores.

The bioassay employing intact cores of sediment allows measurement of toxicant effects under conditions that closely resemble those found in nature. Disturbance of the sediment structure is minimized and by allowing the cores to settle after sampling, variability between replicate cores is much lower than if the bioassay is conducted immediately after sediment sampling. This incubation period presumably allows any disrupted chemical microgradients

to be re-established and the physiological state of the sediment microbes to return to normal. The increased ecological validity of sediment core bioassays over slurry bioassays is offset, to some degree, by the longer time required for the core bioassay to be completed.

In order to achieve high toxicant concentrations in the cores, when a 10 uL aliquot is injected, the toxicant stock solution must be of high concentration. The solubility of some of the metal salts in sea water limits the upper concentration that can be achieved in the toxicant stock solution and subsequently in the cores, without significantly altering the amount of interstitial water in the core. As a result, the calculated EC_{50} values, for some metals, were above the highest toxicant concentrations achieved in the cores. While any sampling method will upset the physical structure and alter the microgradients of natural sediments (Bell and Ahlgren, 1987), the taking of sediment cores appears to be the least disruptive. If environmentally reliable estimates of disposal impacts are to be achieved in the laboratory, sediment microbiota responses to pollutants must be measured in intact sediment cores, to minimize the disturbance of sediment structure. Under these experimental conditions, the use of fluorogenic enzyme substrates appears to offer much promise for toxicity testing of waste materials.

5.5 Bioassays Based on the Incorporation of Labelled Organic Compounds

Thymidine is taken up by nearly all marine bacteria and its rate of incorporation into DNA is well correlated with growth (Pollard & Moriarty, 1984). Thymidine incorporation into DNA has been shown to be more sensitive to contaminants than the uptake of other labelled substrates. This is to be expected as cellular growth is affected by a wider range of metabolic processes than glucose or glutamate uptake (Jonas et al., 1984). Riemann et al. (1987) assumed isotopic dilution of thymidine to be negligible and found isotope incorporation and growth rates difficult to correlate. Using specific activity of $^{32}PO_4$ incorporation into DNA as a measure of DNA synthesis, Fuhrman & Azam (1982) found that thymidine incorporation, without taking into account isotopic dilution, underestimated the rate of DNA synthesis by a factor of 3 to 7. If thymidine incorporation is to be used as an absolute measure of growth rate, the degree to which the incorporated label is diluted by

intracellular pools of thymidine, prior incorporation into DNA (Fuhrman & Azam, 1982), must be determined. Isotopic dilution can be calculated using a range of thymidine concentrations and following their uptake rates in order to establish the size of the intracellular thymidine pool (Pollard & Moriarty, 1984; Findlay et al., 1984). However, the debate concerning isotopic dilution is only of minor significance in pollutant impact assessment since it is usually considered sufficient to measure thymidine incorporation rates relative to a control. Thus, toxicant effects are measured on relative rather than absolute growth rates.

Experimental procedures for the development of two microbial bioassay procedures are based on the uptake of radiolabelled organic compounds, including thymidine and glutamic acid, as described below. The experimental procedures developed as a result of these studies are outlined in Appendix C.

5.5.1 Metal Toxicity Experiments

Objective: To assess radiolabelled organic uptake techniques as bioassay tools in the study of sediment bacteria responses to toxicants.

Methods and Results: The effect of toxic metals on bacterial uptake of ^{14}C-glutamic acid and ^{3}H-thymidine, and the activity of the enzymes, glucosidase, phosphatase and protease were evaluated in slurries of sandy sediment. The methods employed to measure the uptake of radiolabelled compounds are described in Appendix C. In some of the experiments, at higher concentrations of toxicant, values of close to 100% inhibition were observed. In these cases, only data points in the linear portion of the dose-response curve were used in the regression to calculate the EC_{50} values. All calculations were performed using the BASIC microcomputer program EC50.BAS (Appendix D).

In sandy sediment, low EC_{50} values for copper were found in all of the assays (Figs. 17, 18; Table 4) with EC_{50} values for the protease, thymidine and glutamic acid assays below the lowest concentration used (1 ppm). Mercury was found to be extremely toxic in the

glutamic acid and thymidine assays. There were insufficient numbers of points in the linear portion of the dose-response curve to calculate a linear regression. EC_{50} values were well below the lowest metal concentration used. Similarly, with the exception of phosphatase, the enzyme assays showed low EC_{50} values for mercury (Figs. 19, 20; Table 4). Nickel addition resulted in very similar EC_{50} values in all five assays (Figs. 21, 22; Table 4). Cadmium addition caused effects similar to those obtained with mercury (Figs. 23, 24; Table 4). The glutamic acid and thymidine assays with cadimum yielded EC_{50} values that were lower than the enzyme assays. In the bioassay tests with cadmium, the phosphatase assay yielded the highest EC_{50} values. With the exception of protease, following exposure to lead, the glutamic acid and thymidine assays yielded lower EC_{50} values than the enzyme assays in the experiments evaluating the biological effects of lead and zinc (Figs. 25, 26, 27, 28; Table 4).

The toxicity order was similar for all five bioassays, following a general pattern of Cu = Hg > Zn > Ni > Pb = Cd comparable to the trend observed in the previous experiments with sandy sediments.

Conclusions: The uptake of such radiolabelled organic molecules as thymidine and glutamic acid can be used to quantify bacterial responses to environmental toxicants in bioassays for monitoring. In the present study, the uptake of radiolabelled organic substrates appears to be more sensitive than the enzyme bioassays to the range of metals tested. The greater sensitivity of the radioisotope assays is due to the dependence of both glutamic acid metabolism and thymidine assimilation on a wide range of cellular functions. Any interference with cellular metabolism by a toxicant will be reflected in the radioisotope assimilation. Conversely, the exoenzyme bioassays are not dependent on such a wide range of cellular activities; minor disturbances in metabolism may not be detected in these assays.

While the radiolabelled organic bioassays appear to be more sensitive to toxicants, this is offset by the much higher cost of the equipment and materials required. Since toxicity ranking of the six metals tested is similar for all five bioassays, any one of them can be used to assess toxicant impacts on microbial processes in sediments. However, a battery approach of at least three is recommended.

6.0 OCEAN DISPOSAL OF SEDIMENTS: APPLICATION OF BACTERIAL TESTS FOR ENVIRONMENTAL IMPACT ASSESSMENT AND TOXICITY EVALUATION

Harbors and waterways, throughout the world, are dredged for navigation on a regular basis. Most are adjacent to highly industrialized sites where the sediments become contaminated. Contaminated sediment presents a risk to benthic biota in the harbors and at open water dump sites following dredge spoils disposal.

Open water disposal of solid or liquid wastes has become a principal method of waste disposal worldwide, since it is cheaper than landfilling or confined aquatic disposal (Iskandar et al., 1984). Bacterial activity measurements can be used to monitor conditions at specific dump sites, to ascertain conditions before and after waste disposal events, to pre-screen wastes to determine suitability for oceanic disposal, and to assess the likely impact of disposal activities on large-scale processes. Effects of pollutants in an ecosystem depend on the conditions experienced. Differences in parameters such as organic carbon concentrations can greatly affect pollutant toxicity (Cunningham et al., 1986). Toxicity testing of any disposal event must be conducted under conditions as close as possible to those at the proposed dump site.

No single test will be suitable in all situations (Coleman & Qureshi, 1985; Vives-Rego et al., 1986) and it is advisable to employ a battery of tests in disposal impact assessment. Furthermore, use of a wide spectrum of tests increases reliability and ease of interpretation of results in situations where sediment heterogeneity or differences in exposure routes may cause ambiguities. The choice of which tests should be used depends on time, data requirements, biological and cost considerations (Williams et al., 1986). Sayler et al. (1982) used a minimum of nine tests to assess the pollution status of marine sediments and reported that viable plate counts, ATP concentrations, phosphatase activity, naphthalene and phenanthrine mineralization rates were good indicators of sediment contamination. Lipid and glucose mineralization, nitrogen fixation and sediment protein concentrations were judged to be acceptable indicators of contaminant induced stress, while anaerobic population density and methanogenesis were not. More recently, a Sediment Quality Triad

approach, based on the concurrent measurement of sediment chemical contamination, changes in resident infauna community structure and toxicity, has been proposed as the ideal means of assessing marine pollution (Chapman et al., 1987).

In comparison to other commonly employed bioassays, the tests developed in this project are sensitive (Table 8) with EC_{50} values comparable to those obtained in fish bioassays and lower than those obtained in Microtox bioassays. Furthermore, the sampling of natural sediment allows measurement of toxicant impacts in an aquatic community, in contrast to a single species of questionable relationship to the environment under study. However, if routine monitoring of effluents or other wastes is desired, these procedures can be modified into a standardized toxicity test by using lyophilized strains of a "standard" bacterium as the test "subject". The use of a single bacteria strain would greatly reduce the variability between tests, facilitate intra- and inter-laboratory calibration, and eliminate the need to collect sediment samples in the field for routine testing.

It is essential that any test designed to predict toxicant impacts on the environment retains as much similarity to natural conditions as possible. While bacteria in sediment slurries appear to be less sensitive to toxicants (shown by the generally lower EC_{50} values observed in experiments using intact cores), the results of our study suggest that when toxicity information is required urgently, bioassays employing sediment slurries with MUF-substrates can be used. If more sensitive and environmentally valid toxicity estimates are required bioassays employing intact sediment cores can be used.

Following oceanic disposal of sewage sludge, it has been reported that oxygen consumption rates increased far above the background in both the water column and sediment. However, consumption rates quickly returned to normal after 3-6 h in the water column (Thomas & O'Reilley, 1981) and after 2 d in the sediment (Phoel et al., 1981). Similarly the effects of coal combustion residues lasted only 1-2 days (Rose et al., 1981). These findings illustrate the fact that the effects of oceanic waste disposal tend to be short lived. In order to accurately assess disposal impacts, it is imperative that short-term assays, such as those developed in the present study, be used. Long-term studies, using these procedures also have relevance for evaluation of the effects of contaminants. They provide information

about microbial processes which are sensitive to the perturbing agent, and concentrations and durations of effect.

At present the use of radiolabelled organic substrates and fluorogenic enzyme substrates in bioassay tests appears to offer the most promise in toxicity testing of waste materials. For routine monitoring of dump sites or effluents, microbial bioassays may be used for primary screening of wastes to indicate if further tests with higher levels of diversity are required. Besides being sensitive, they are faster, easier and less costly to perform than fish or invertebrate assays.

7.0 SUMMARY

Sediments are effective sinks of contaminants, therefore, chemical analysis of a given area reflects degree and nature of contamination. However, the resulting chemical data provide little or no evidence of biological consequences of the contamination. As a result, there exists a great need for simple and cost effective tests of toxicity for the waste materials disposed in the sea (Buikema et al., 1980; Busch, 1982).

Traditionally, toxicant effects on bacterial activity were followed by measurement of growth rates, oxygen consumption or colony formation on agar plates (Liu, 1981). Early studies of bacterial effects of waste disposal primarily concerned the introduction of foreign species, such as E. coli (Graikoski, 1981). At present the major concern in the development of toxicity assessment procedures is their ecological validity (Laskowski-Hoke & Prater, 1981).

Laboratory toxicity assays must incorporate ecological principles into their design (Neuhold, 1986). Bulk chemical analyses and studies of community structure are insufficient to accurately predict the impact of wastes in the environment. In order to gain complete understanding of the environmental effects of contaminants, effects must be examined at the ecosystem level, not just the population and community level. The focus should be on primary metabolic processes since a disturbance of these processes will have ramifications throughout the ecosystem. Assays should be conducted with indigenous biota

in the soil or water receiving the wastes in order to obtain ecologically valid evidence on the potential damage caused by contaminants to the environment (Bauer et al., 1981; Chapman & Long, 1983). The most accurate laboratory predictions of toxicant impact on sediments are achieved with tests conducted under ambient conditions using intact sediment cores.

The use of methylumbelliferyl (MUF) and radiolabelled substrates to assess exoenzyme activity in sediments yields rapid, inexpensive and sensitive bioassays that have many advantages over conventional sediment tests. Methylumbelliferyl enzyme substrate analogues are available for a wide range of exoenzyme systems, thus a specific substrate can be chosen for many basic processes, such as nutrient cycling, carbohydrate or protein degradation. The sensitivity of these enzyme bioassays is similar to that shown by other sediment bioassays using chromogenic enzyme substrates and radiolabelled organic incorporation. The advantages are that the fluorogenic substrates require much shorter incubation periods, are used at much lower concentrations than chromogenic substrates, and are much less expensive than radiolabelled organic substrates. Traditional bioassay tests using bacteria based on the measurement of growth rates, oxygen consumption, or colony formation (Liu, 1981) require lengthy incubations during which a single concentration of toxicant is exposed to increasing biomass (Trevors et al., 1981). These long periods of incubation also increase the risk of selecting resistant forms and, thus, effects of a toxicant on these forms, not the whole population, are measured (Barhart & Vestal, 1983). The new bioassay procedures described are based on the use of relatively short incubation periods.

It is impossible to fully assess the impact of any polluting agent on the biological activity and chemistry of an impacted area without taking into account effects on microbial activity (Griffiths, 1983). The assay procedures developed can be used both as standard toxicity bioassays and as tools for monitoring long-term effects of contaminants on marine environmental quality.

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10.0 TABLES

Table 1: The toxicity of six metals to glucosidase, phosphatase, and protease in slurries of sandy sediment. All values are mg/L.

Glucosidase

Metal	EC_{50}	95% Confidence Interval
Cu	41	16 -> 107
Hg	27	3 -> 242
Ni	130	42 -> 407
Cd	72	63 -> 81
Pb	129	51 -> 326
Zn	29	5 -> 164

Phosphatase

Metal	EC_{50}	95% Confidence Interval
Cu	9	1 -> 63
Hg	>100	-
Ni	173	44 -> 323
Cd	439	92 -> 2112
Pb	395	112 -> 1396
Zn	87	37 -> 204

Protease

Metal	EC_{50}	95% Confidence Interval
Cu	<1	-
Hg	>100	-
Ni	112	25 -> 493
Cd	288	72 -> 1146
Pb	33	12 -> 92
Zn	40	13 -> 118

Table 2: The toxicity (EC_{50}) of six metals to glucosidase activity in marine sediments using fluorogenic substrates (this study), and freshwater sediments using colourimetric substrates (Barnhart & Vestal, 1983). All values are mg/L.

Metal	Marine Sediment	Freshwater Sediment
Cu	41	10
Hg	27	26
Ni	130	60
Cd	72	100
Pb	129	--
Zn	29	100

Table 3: The toxicity of six metals to glucosidase, phosphatase, and protease activity in slurries of muddy sediment. All values in mg/L.

Glucosidase

Metal	EC_{50}	95% Confidence Interval
Cu	35	<1 -> 2982
Hg	35	10 -> 121
Ni	310	236 -> 406
Cd	316	32 -> 3150
Pb	>1000	-
Zn	76	28 -> 207

Phosphatase

Metal	EC_{50}	95% Confidence Interval
Cu	5	<1 -> 193
Hg	28	4 -> 229
Ni	232	39 -> 1375
Cd	95	31 -> 234
Pb	769	133 -> 4446
Zn	58	32 -> 106

Protease

Metal	EC_{50}	95% Confidence Interval
Cu	10	1 -> 107
Hg	70	12 -> 406
Ni	120	86 -> 168
Cd	198	9 -> 4447
Pb	51	17 -> 153
Zn	25	12 -> 51

Table 4: The toxicity of six metals to glucosidase, phosphatase and protease activity and glutamic acid and thymidine incorporation. All values in mg/L.

Copper

Assay	EC_{50}	95% Confidence Interval
Glucosidase	5	<1 -> 151
Phosphatase	11	2 -> 65
Protease	<1	-
Thymidine	<1	-
Glutamic Acid	<1	-

Mercury

Assay	EC_{50}	95% Confidence Interval
Glucosidase	14	1 -> 315
Phosphatase	130	2 -> 9604
Protease	18	9 -> 37
Thymidine	<1	-
Glutamic Acid	<1	-

Nickel

Assay	EC_{50}	95% Confidence Interval
Glucosidase	310	56 -> 1711
Phosphatase	196	152 -> 252
Protease	130	76 -> 226
Thymidine	241	25 -> 2316
Glutamic Acid	346	12 -> 10000

Table 4: (Cont'd)

Cadmium

Assay	EC_{50}	95% Confidence Interval
Glucosidase	231	29 -> 1880
Phosphatase	999	690 -> 1421
Protease	484	35 -> 6985
Thymidine	144	27 -> 752
Glutamic Acid	100	41 -> 245

Lead

Assay	EC_{50}	95% Confidence Interval
Glucosidase	407	10 -> 16568
Phosphatase	671	73 -> 6617
Protease	33	12 -> 88
Thymidine	105	44 -> 249
Glutamic Acid	113	32 -> 400

Zinc

Assay	EC_{50}	95% Confidence Interval
Glucosidase	33	23 -> 46
Phosphatase	67	19 -> 239
Protease	24	10 -> 54
Thymidine	2	<1 -> 57
Glutamic Acid	12	<1 -> 597

Table 5: The effect of zinc on protease activity in sediment cores. Values are mg/L.

Horizon	EC_{50}	95% Confidence Interval
0-1cm	5	<1 -> 247
1-2cm	2	<1 -> 53
2-3cm	0.9*	-
3-4cm	0.8*	-
4-5cm	3	<1 -> 27
5-6cm	2	<1 -> 18

* Calculated value of EC_{50} outside range of toxicant concentrations used.

Table 6: Horizontal diffusion of ^{14}C-glutamic acid through cores of sandy sediment. Values are DPM +/- S.D. in each concentric ring of sediment.

	Distance from Core Centre (mm)		
Horizon	0-1.5	1.5-4	4-6.3
0-1cm	29000±2000	17000±5000	7000±4000
1-2cm	17800±7800	17000±3800	6100±2000
2-3cm	29800±13000	21300±7300	6000±3000
3-4cm	27000±10000	20200±1700	4900±1900

Table 7: The toxicity of metals to glucosidase activity in cores of sandy sediment. All values are mg/L.

Copper

Horizon	EC_{50}	95% Confidence Interval
0-1cm	74*	-
1-2cm	86*	-
2-3cm	67*	-
3-4cm	42*	-
4-5cm	23*	-
5-6cm	8	1 -> 107

Mercury

Horizon	EC_{50}	95% Confidence Interval
0-1cm	1.3	0.3 -> 5.2
1-2cm	1.3	0.4 -> 3.7
2-3cm	0.5	0.1 -> 1.8
3-4cm	1.4	0.3 -> 7.3
4-5cm	1.3	0.5 -> 3.4
5-6cm	1.0	0.1 -> 8.6

Nickel

Horizon	EC_{50}	95% Confidence Interval
0-1cm	38	5 -> 288
1-2cm	40	2 -> 714
2-3cm	33	7 -> 159
3-4cm	49	3 -> 838
4-5cm	48	7 -> 367
5-6cm	52	6 -> 443

Table 7: (Cont'd)

Cadmium

Horizon	EC_{50}	95% Confidence Interval
0-1cm	1650*	-
1-2cm	1744*	-
2-3cm	4022*	-
3-4cm	1524*	-
4-5cm	1242*	-
5-6cm	4809*	-

Lead

Horizon	EC_{50}	95% Confidence Interval
0-1cm	2360*	-
1-2cm	373*	-
2-3cm	1987*	-
3-4cm	22014*	-
4-5cm	20174*	-
5-6cm	3754*	-

Zinc

Horizon	EC_{50}	95% Confidence Interval
0-1cm	108	10 -> 1172
1-2cm	107	4 -> 2570
2-3cm	77	<1 -> 14375
3-4cm	80	<1 -> 42072
4-5cm	77	1 -> 9547
5-6cm	94	3 -> 3023

* Calculated EC_{50} outside range of toxicant concentrations used.

Table 8: Comparison of results (EC_{50}) of Glucosidase, Thymidine, Microtox and Fish bioassays. Values in mg/L.

Metal	Gluc.	Thym.	Microtox*	Fish*
Cu	5	<1	25	0.1 - 11
Hg	14	<1	0.06	0.01 - 0.9
Ni	310	241	22900	-
Cd	231	144	416	1.0 - 100
Pb	407	173	-	-
Zn	33	<1	476	0.24 - 7

Gluc. = Glucosidase Bioassay, Thym. = ^{3}H-Thymidine Bioassay

* McFeters et al. (1983).

11.0 FIGURES

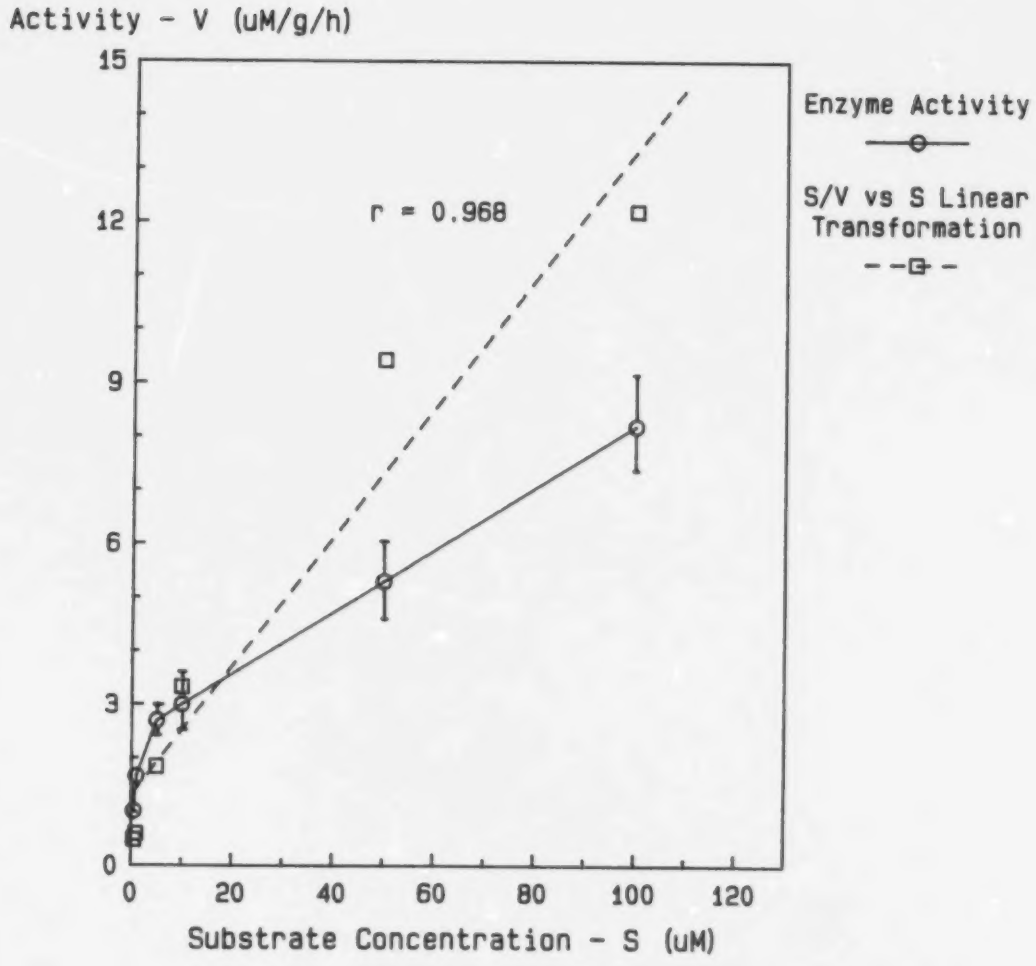

Figure 1: Michaelis-Menten kinetics of B-glucosidase activity in slurries of sandy sediment.

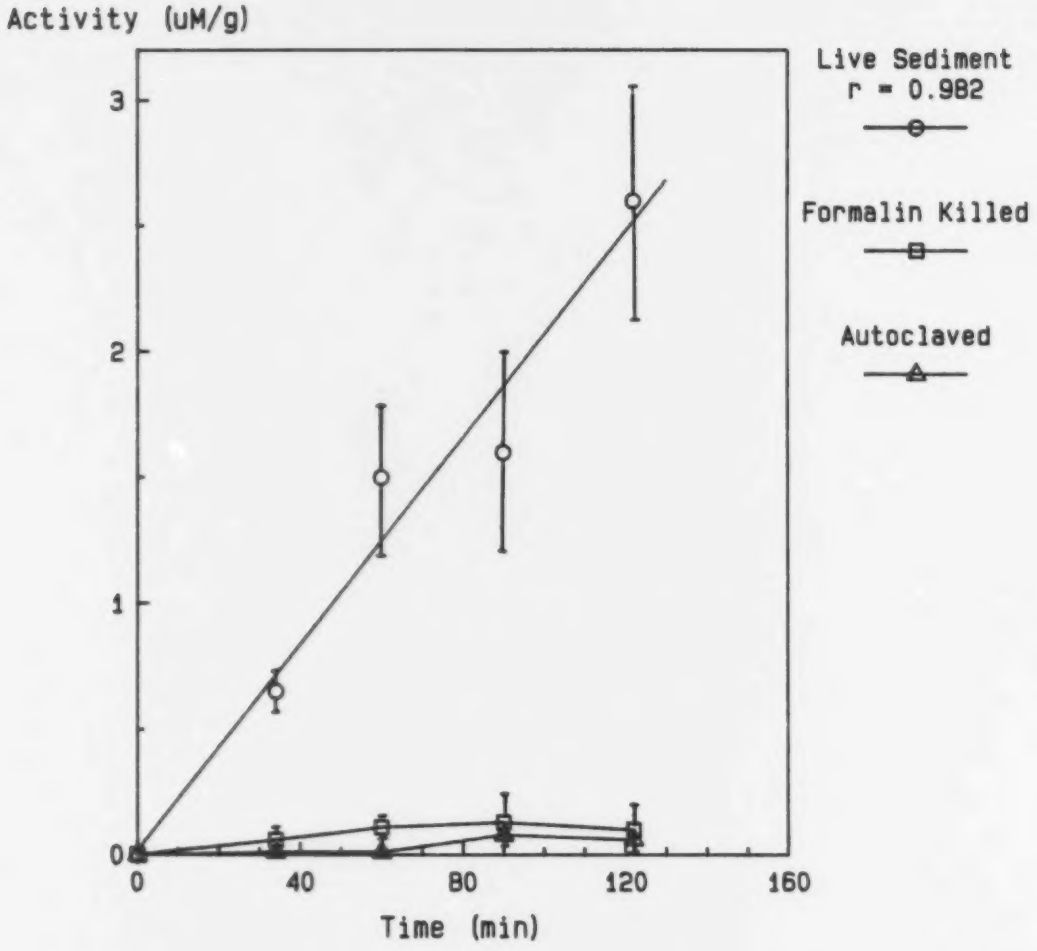

Figure 2: B-glucosidase activity in slurries of sandy sediments over time in live, formalin killed and autoclaved sediments.

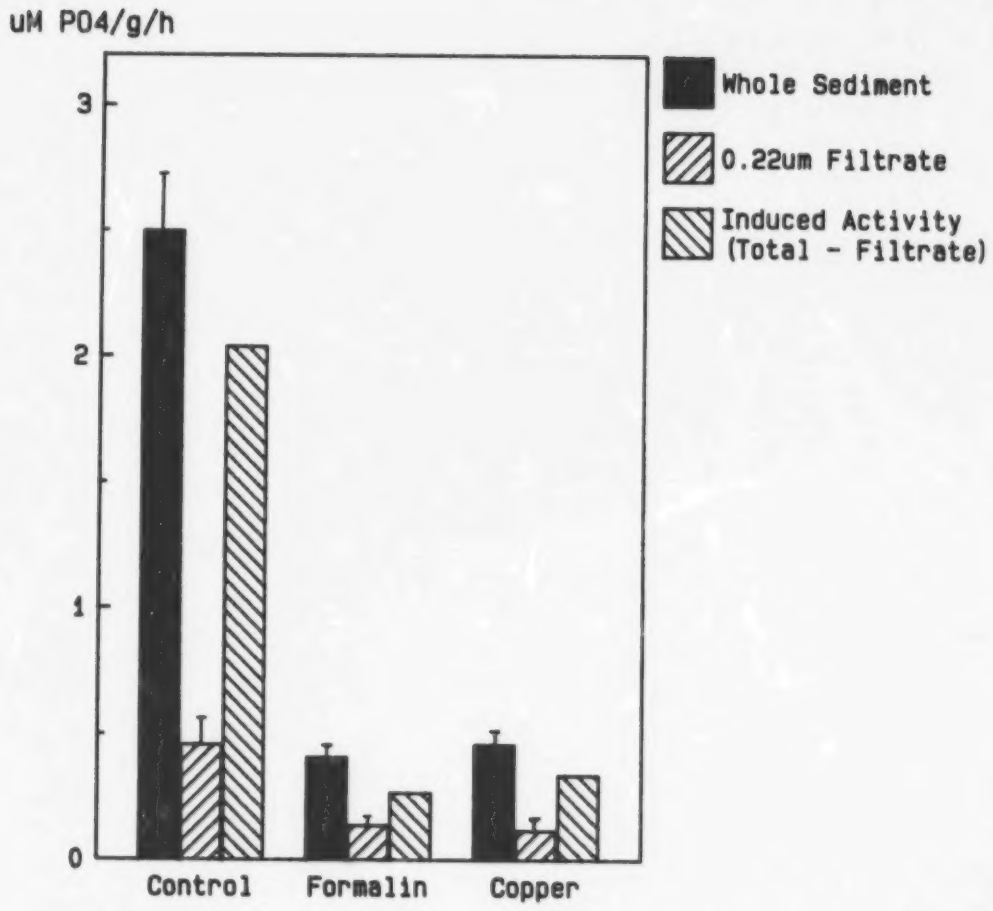

Figure 3: Induced and background phosphatase activity in slurries of sandy sediment exposed to copper and formalin.

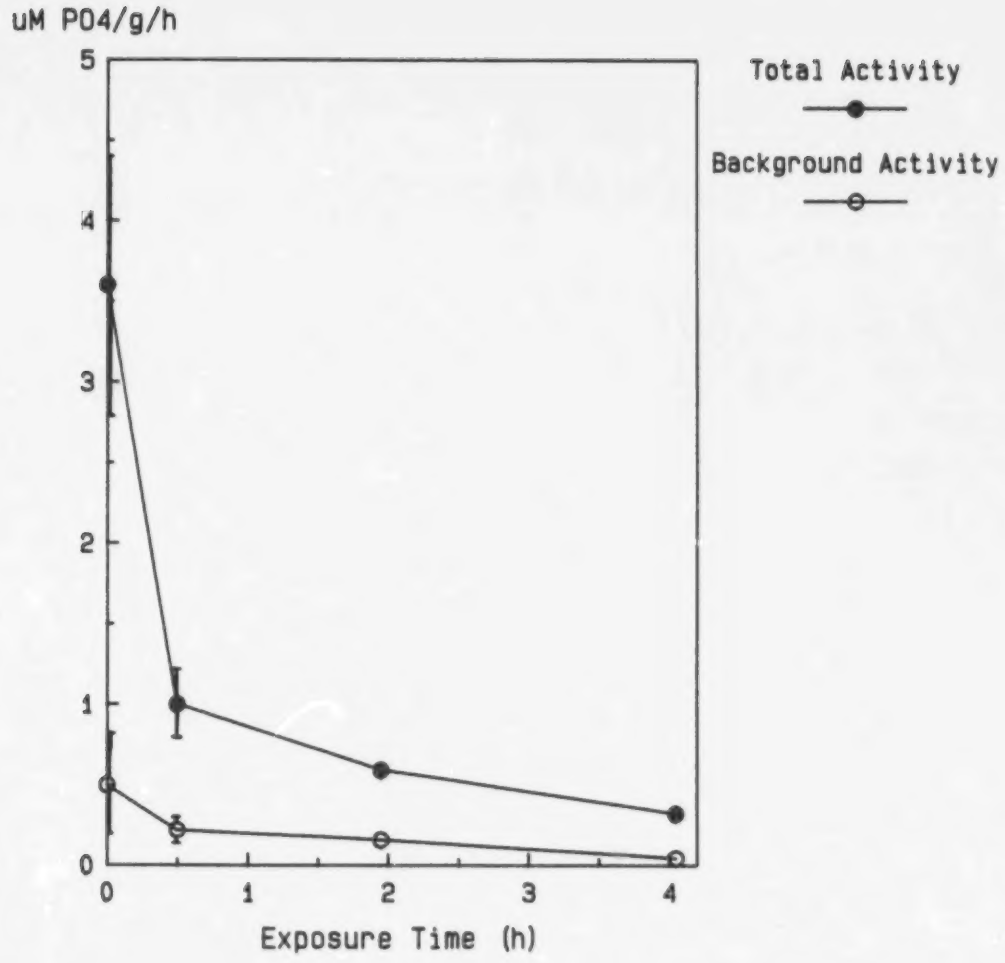

Figure 4: Effect of preincubation time with cadmium on phosphatase activity in slurries of sandy sediment.

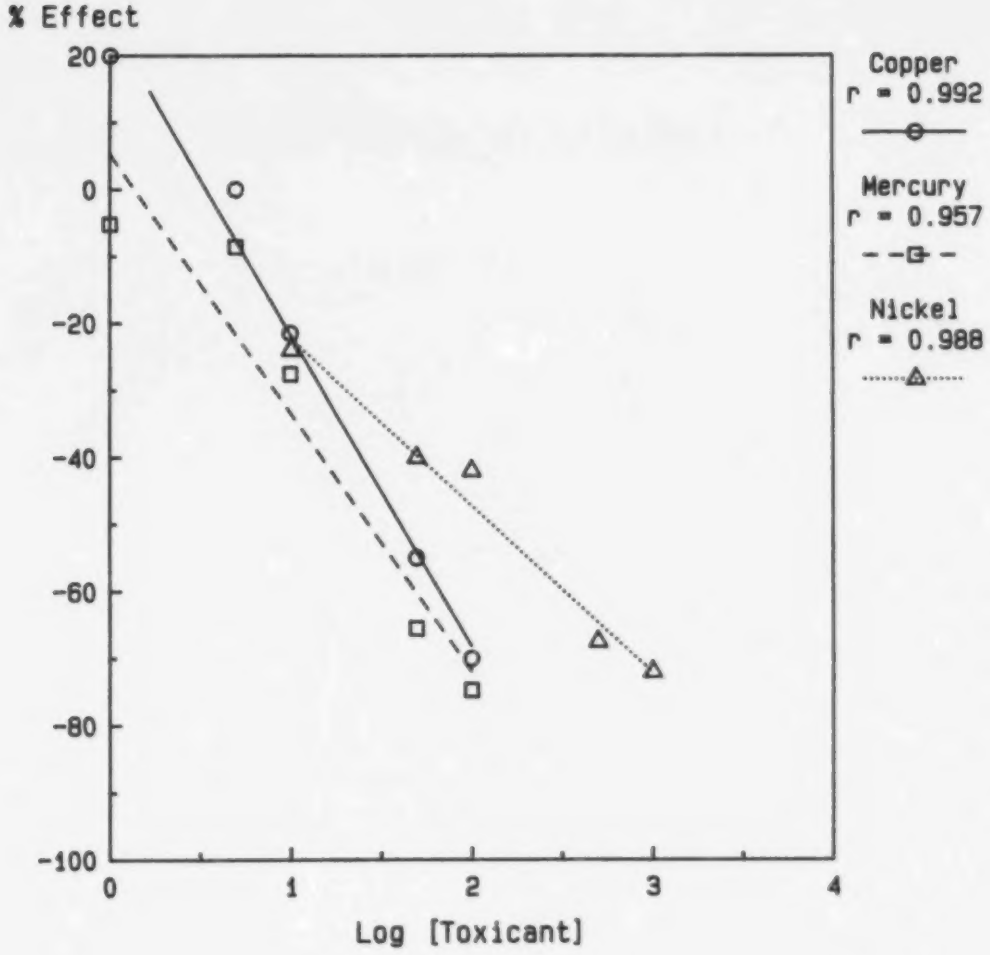

Figure 5: The effects of copper, mercury and nickel on glucosidase activity in slurries of sandy sediment.

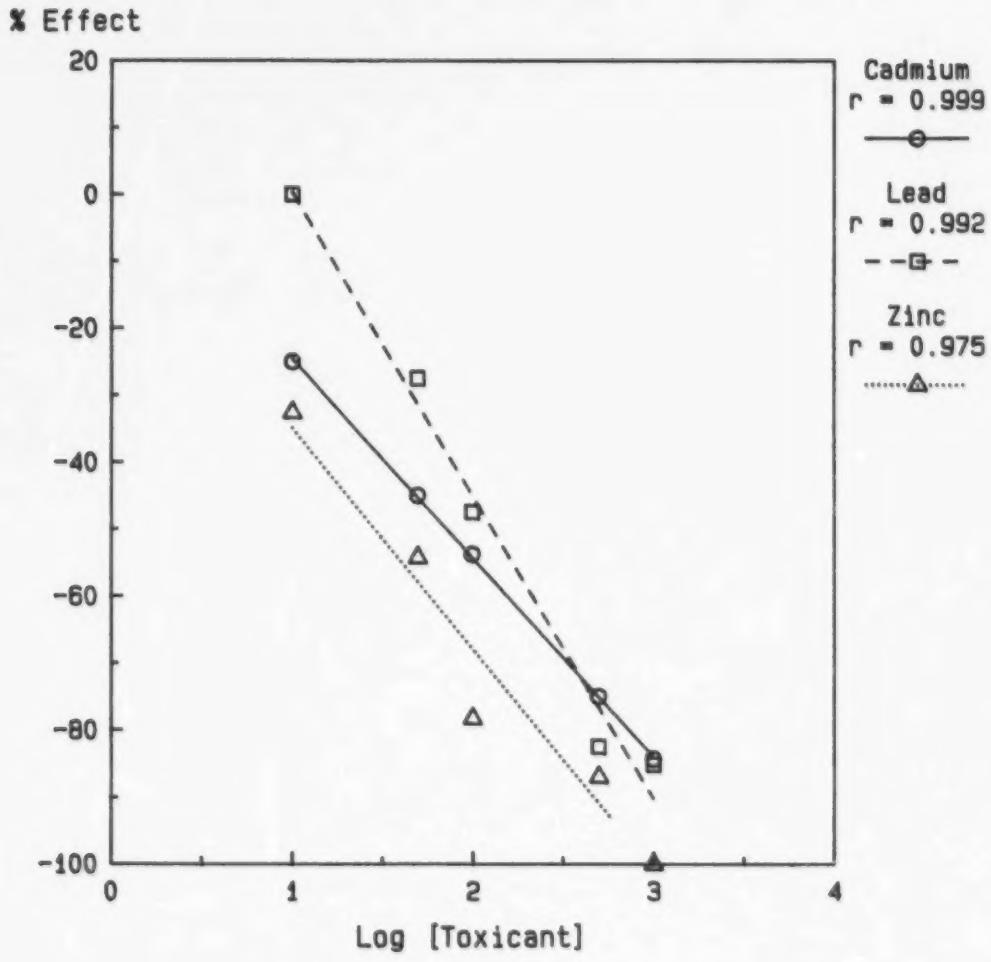

Figure 6: Effects of cadmium, lead and zinc on glucosidase activity in slurries of sandy sediment.

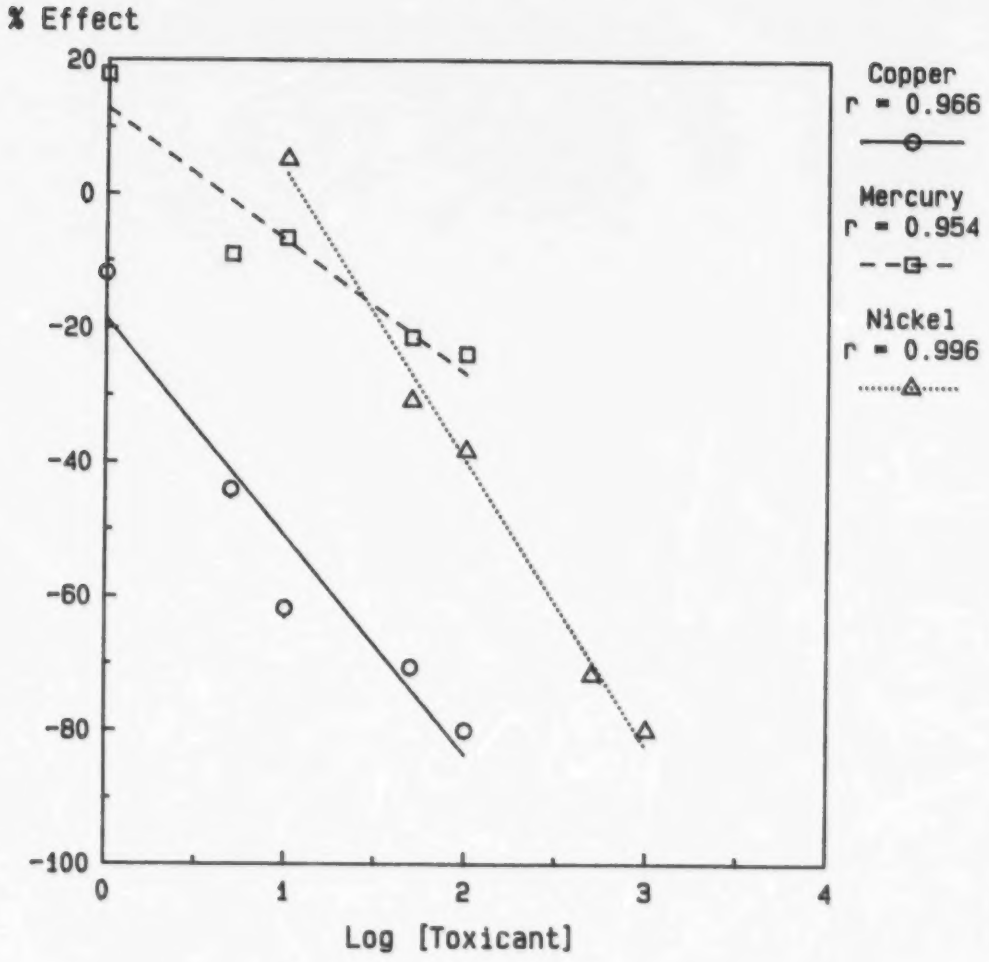

Figure 7: The effects of copper, mercury and nickel on phosphatase activity in slurries of sandy sediment.

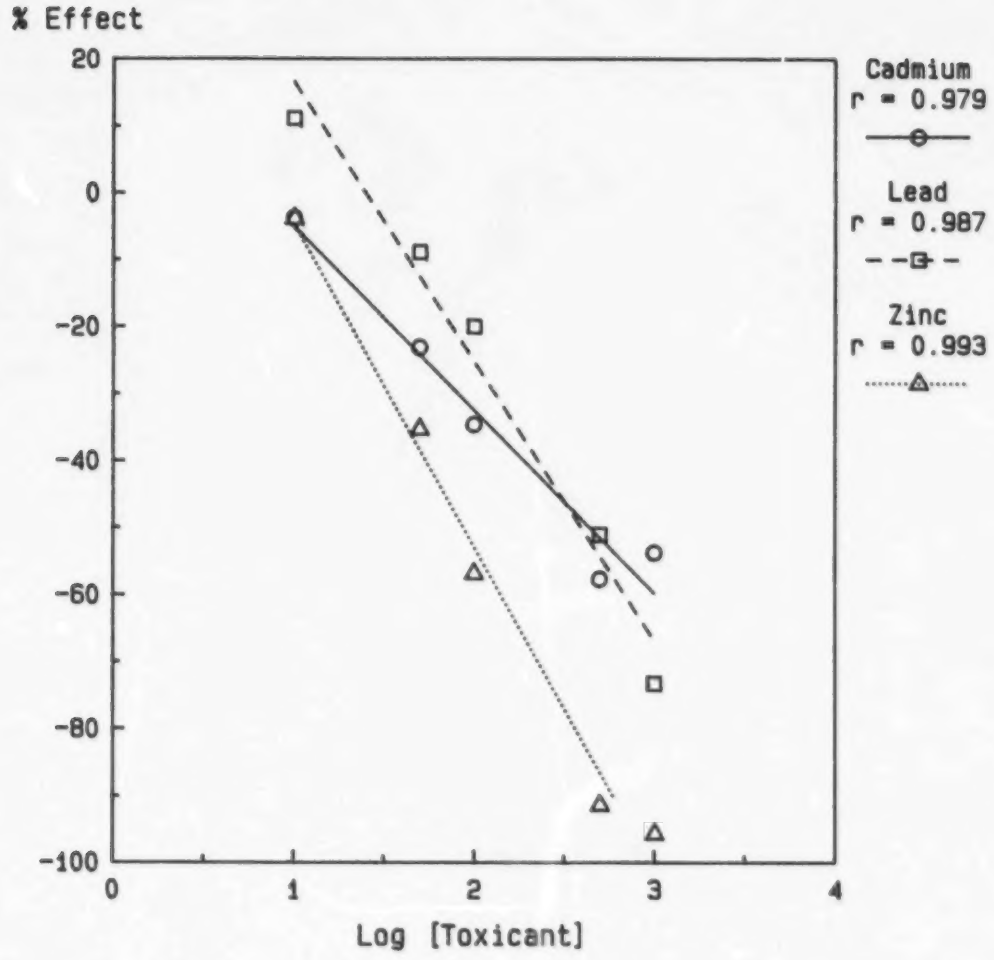

Figure 8: The effects of cadmium, lead and zinc on phosphatase activity in slurries of sandy sediment.

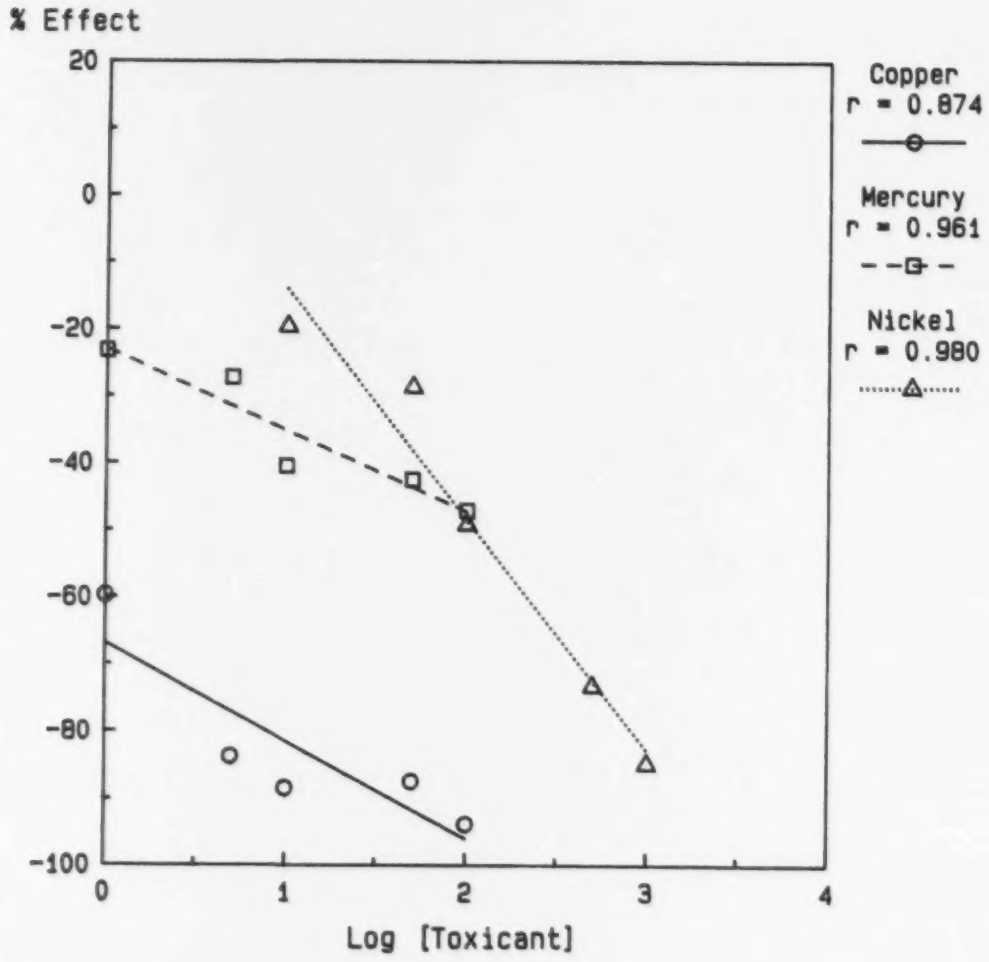

Figure 9: The effects of copper, mercury and nickel on protease activity in slurries of sandy sediment.

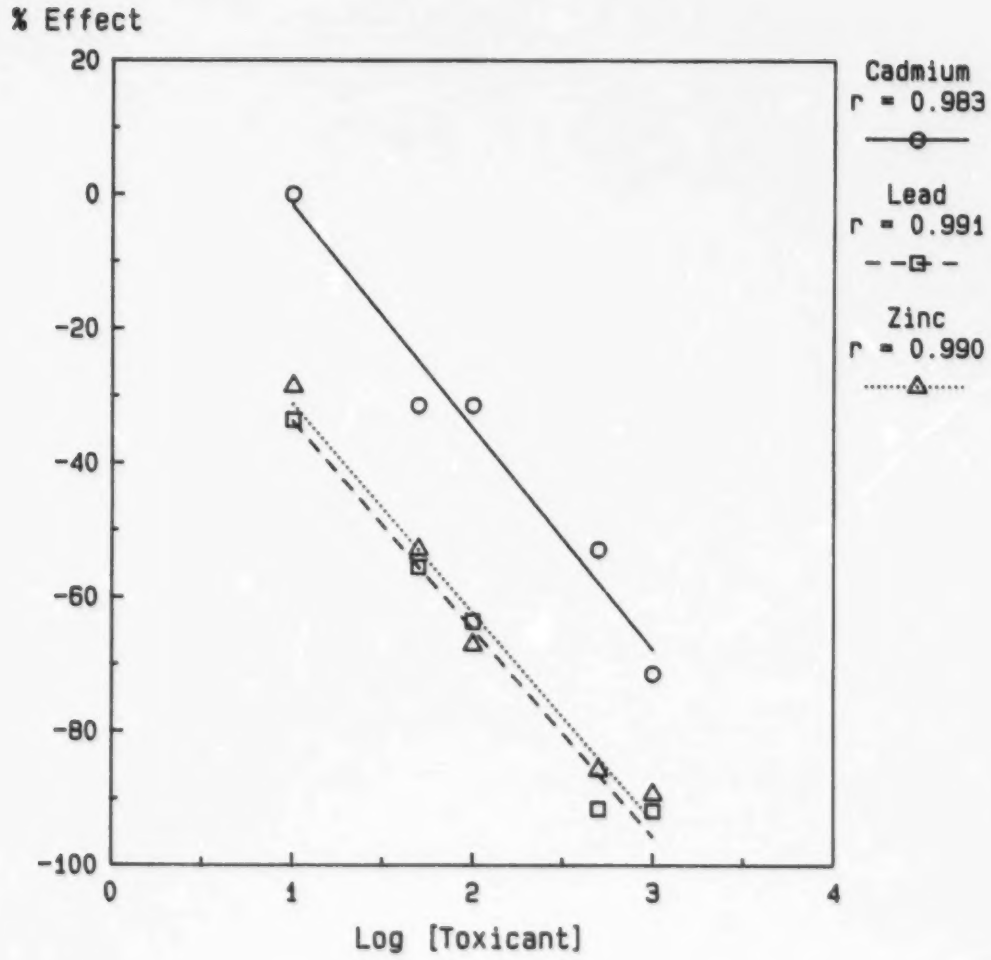

Figure 10: The effects of cadmium, lead and zinc on protease activity in slurries of sandy sediment.

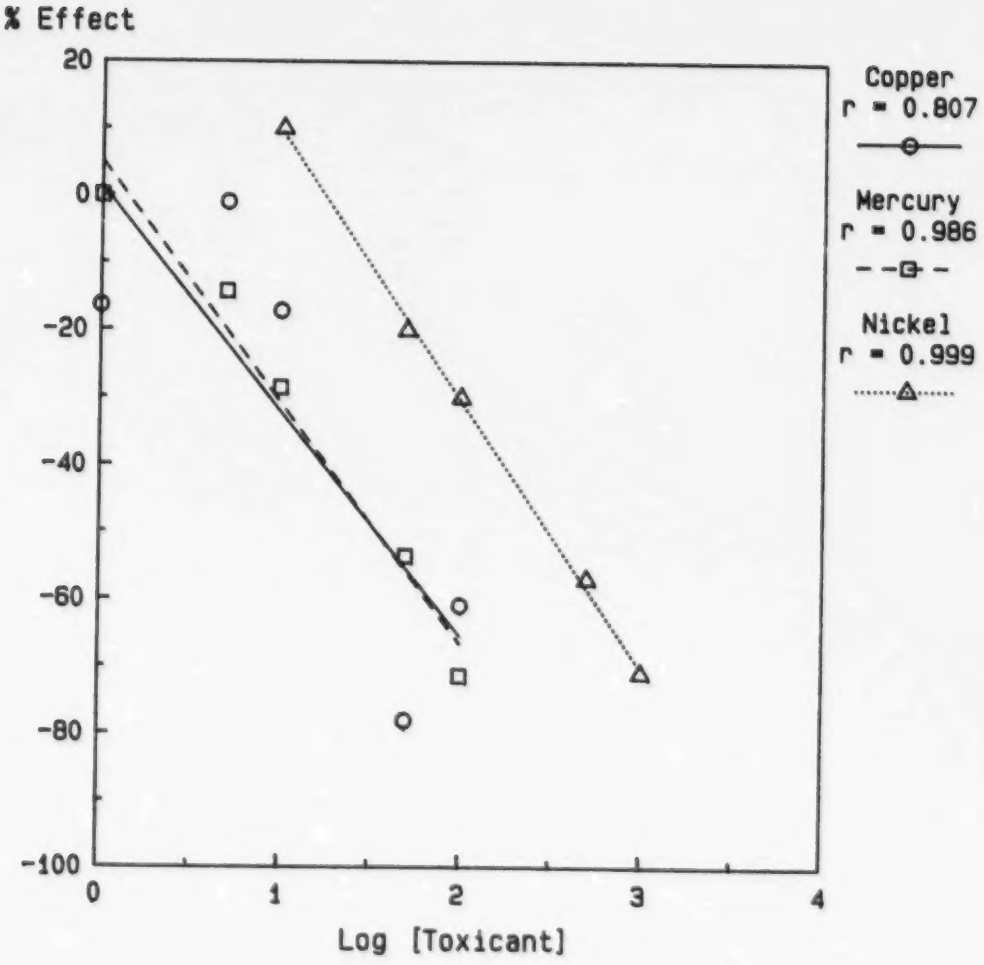

Figure 11: The effects of copper, mercury, and nickel on glucosidase activity in slurries of muddy sediment.

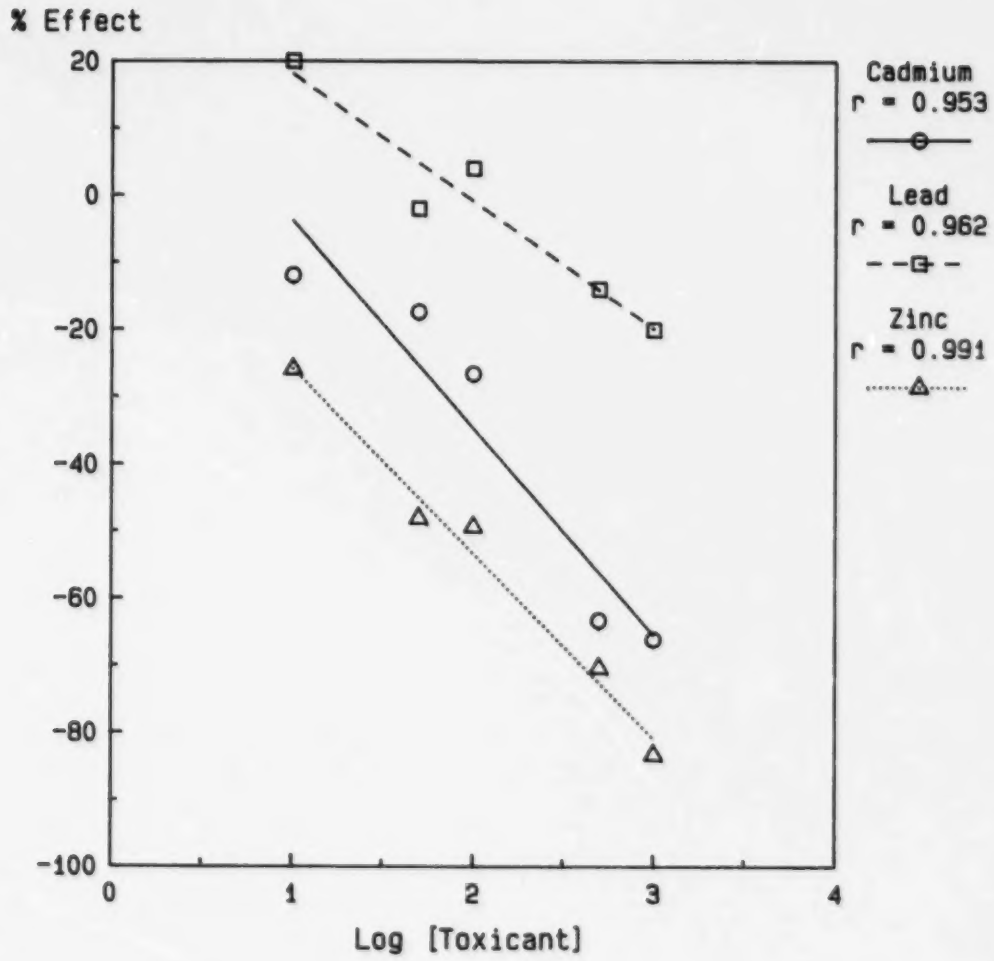

Figure 12: The effects of cadmium, lead and zinc on glucosidase activity in slurries of muddy sediment.

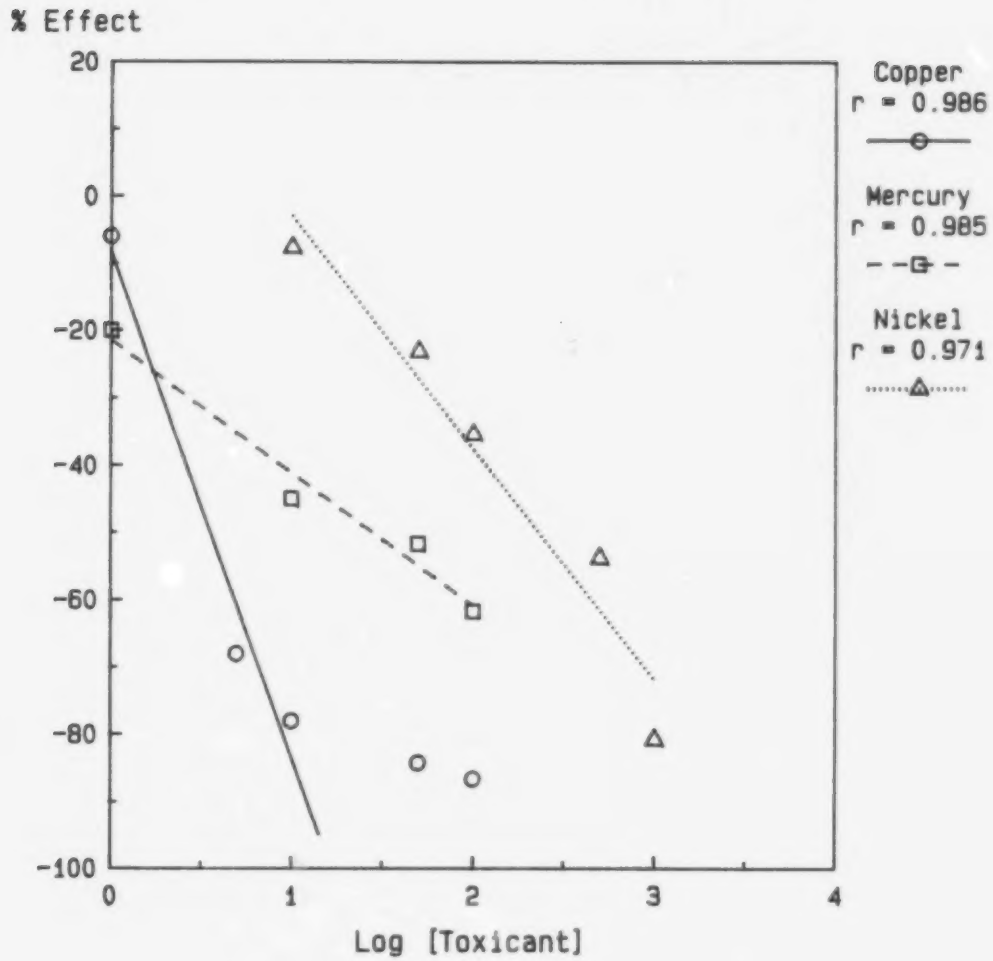

Figure 13: The effects of copper, mercury and nickel on phosphatase activity in slurries of muddy sediment.

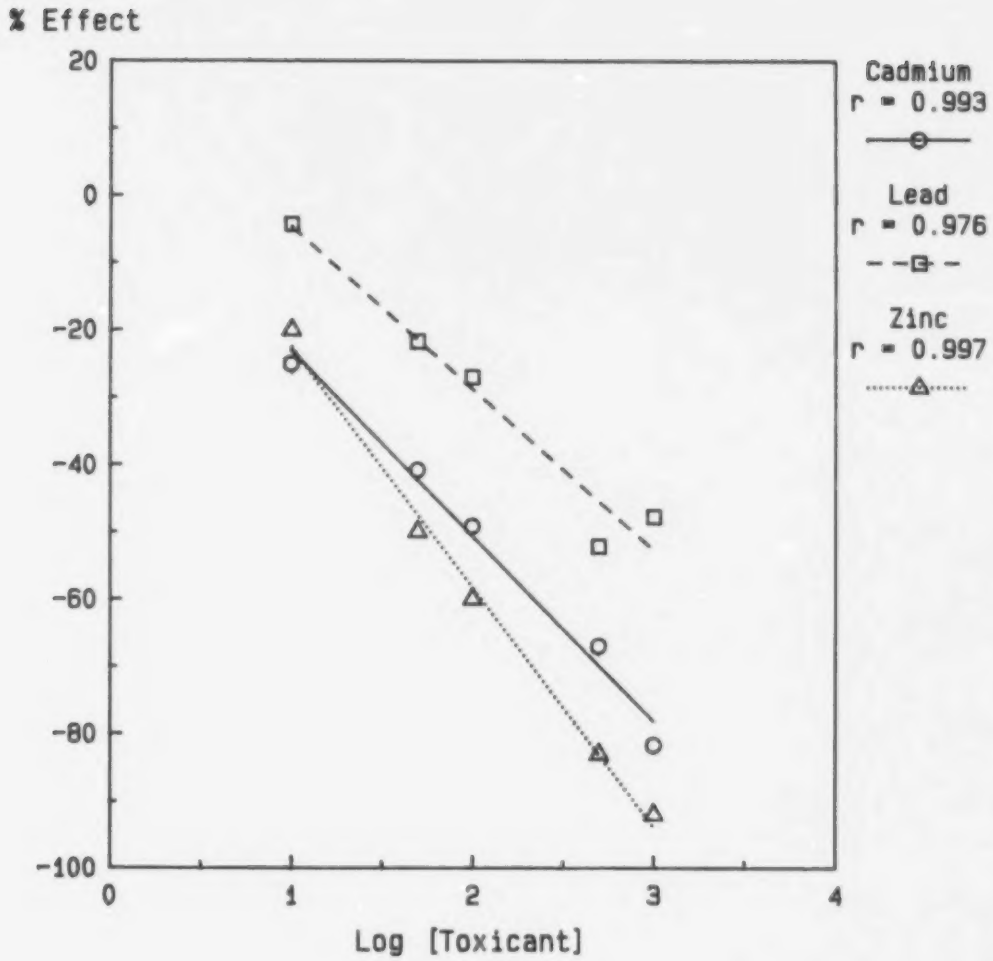

Figure 14: The effects of cadmium, lead and zinc on phosphatase activity in slurries of muddy sediment.

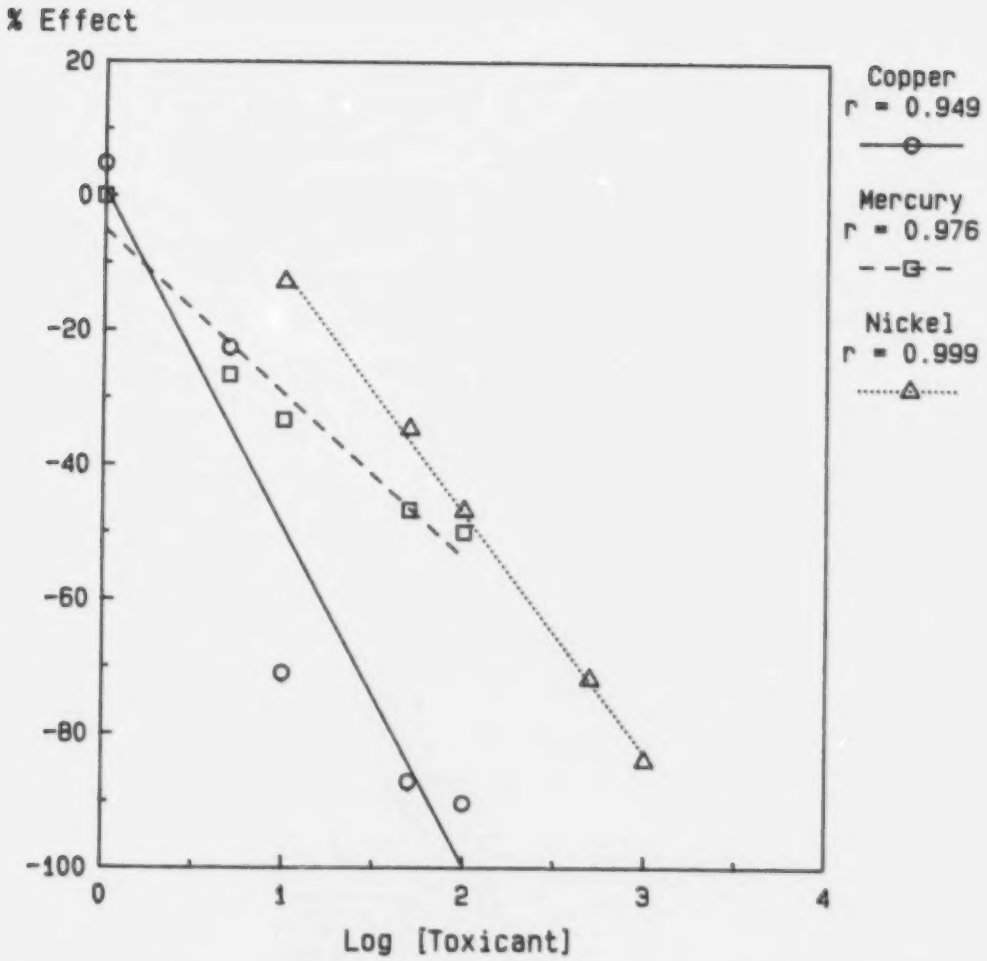

Figure 15: The effects of copper, mercury and nickel on protease activity in slurries of muddy sediment.

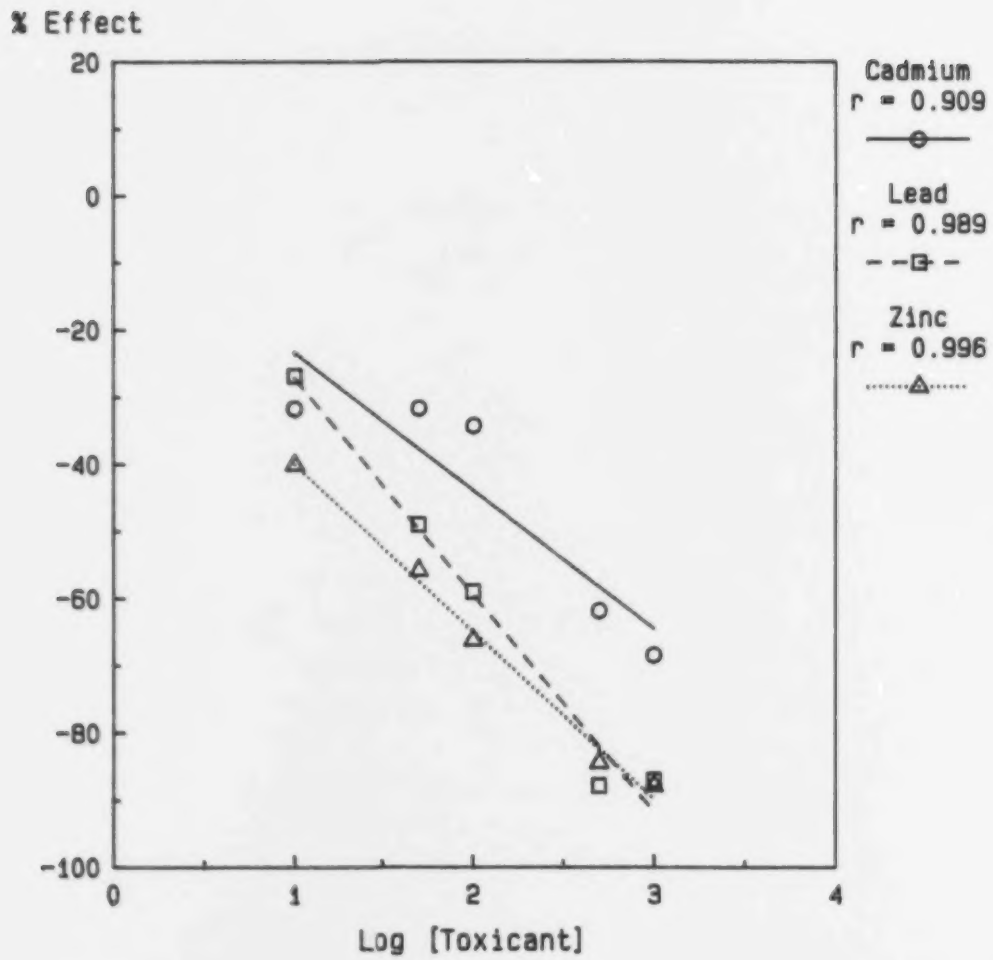

Figure 16: The effects of cadmium, lead and zinc on protease activity in slurries of muddy sediment.

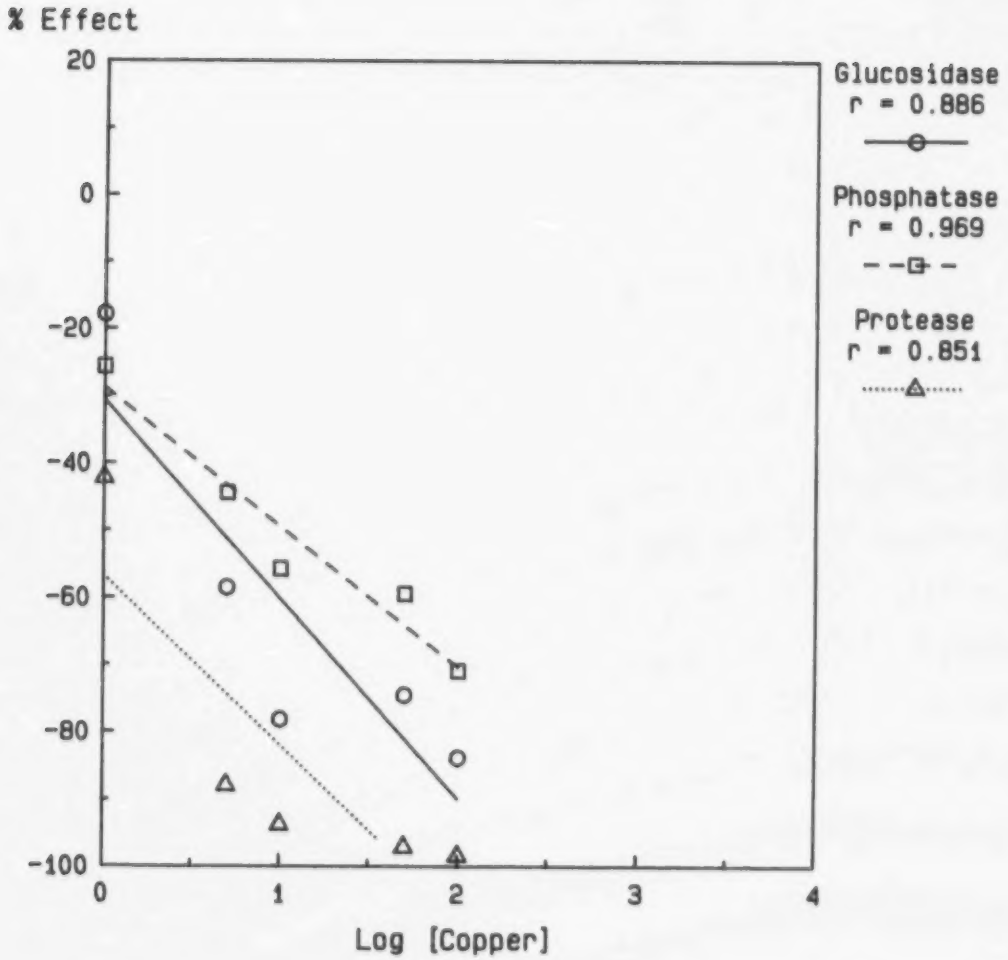

Figure 17: The effects of copper on exoenzyme activity in slurries of sandy sediment.

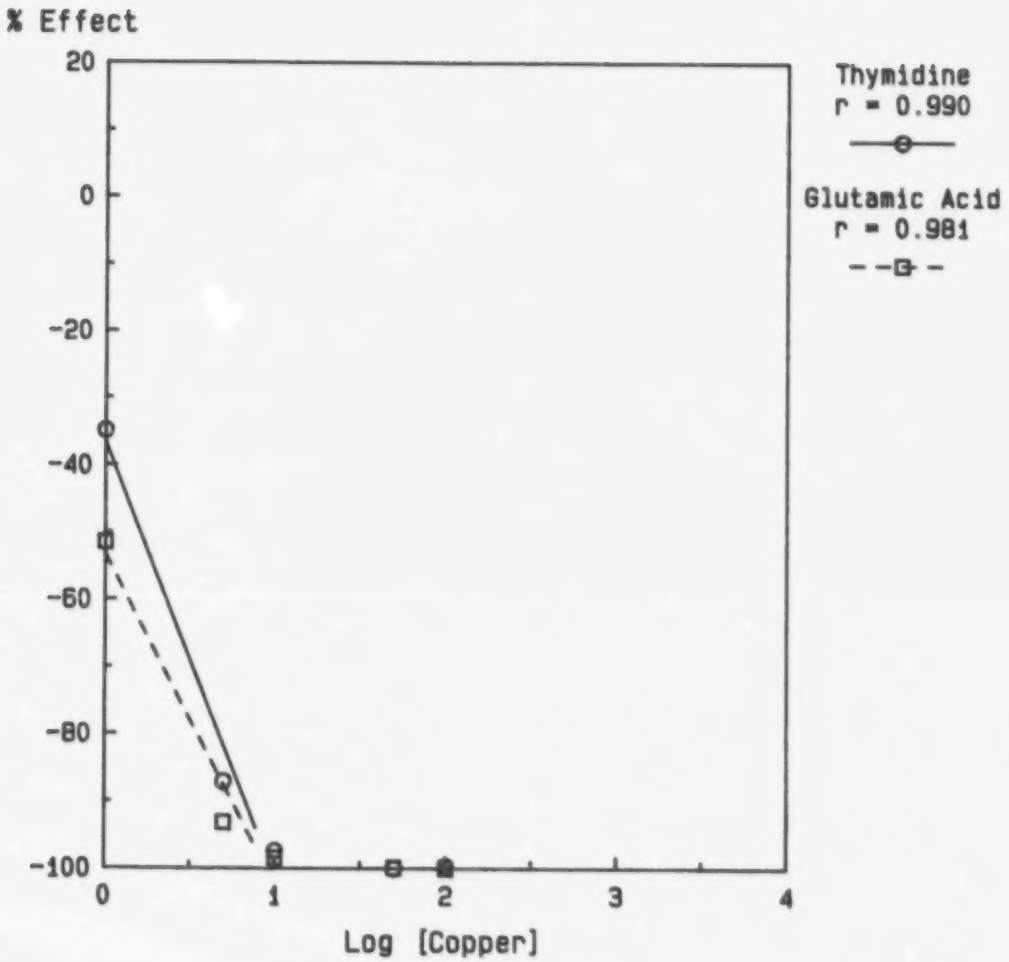

Figure 18: The effects of copper on ^{14}C-glutamic acid and ^{3}H-thymidine uptake in slurries of sandy sediment.

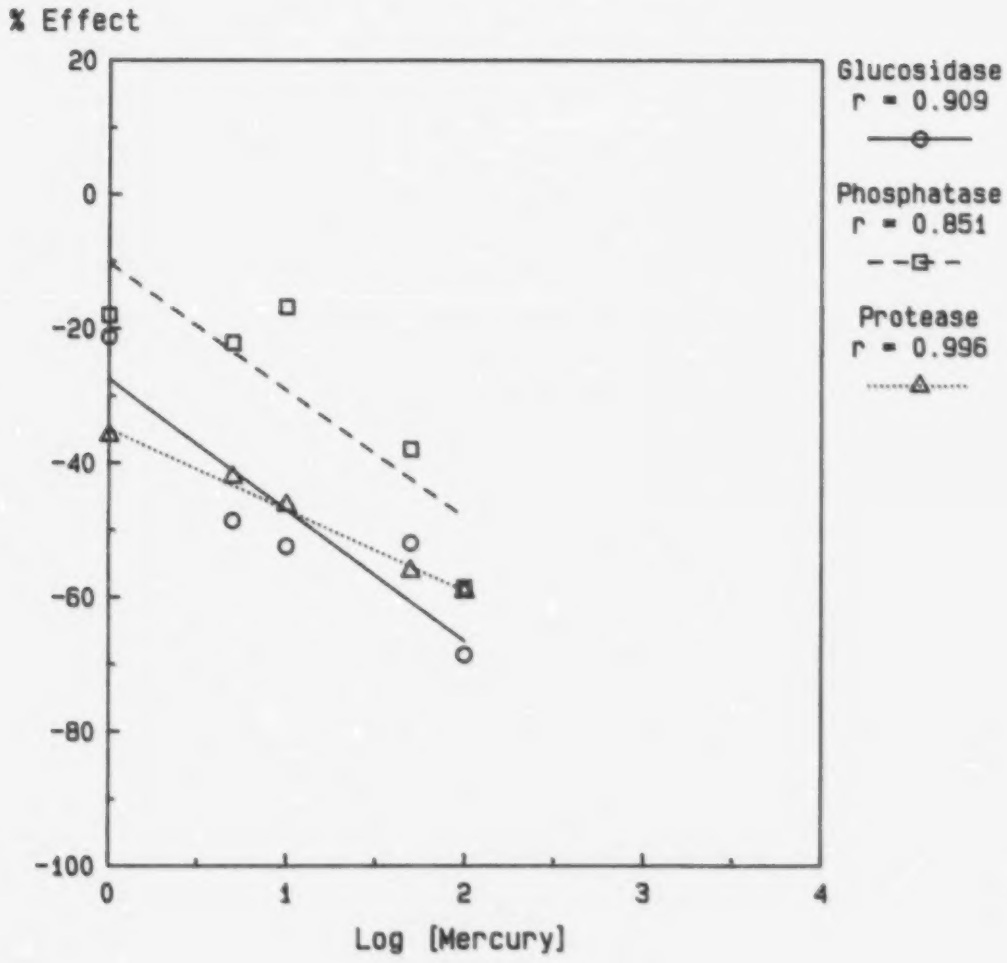

Figure 19: The effects of mercury on exoenzyme activity in slurries of sandy sediment.

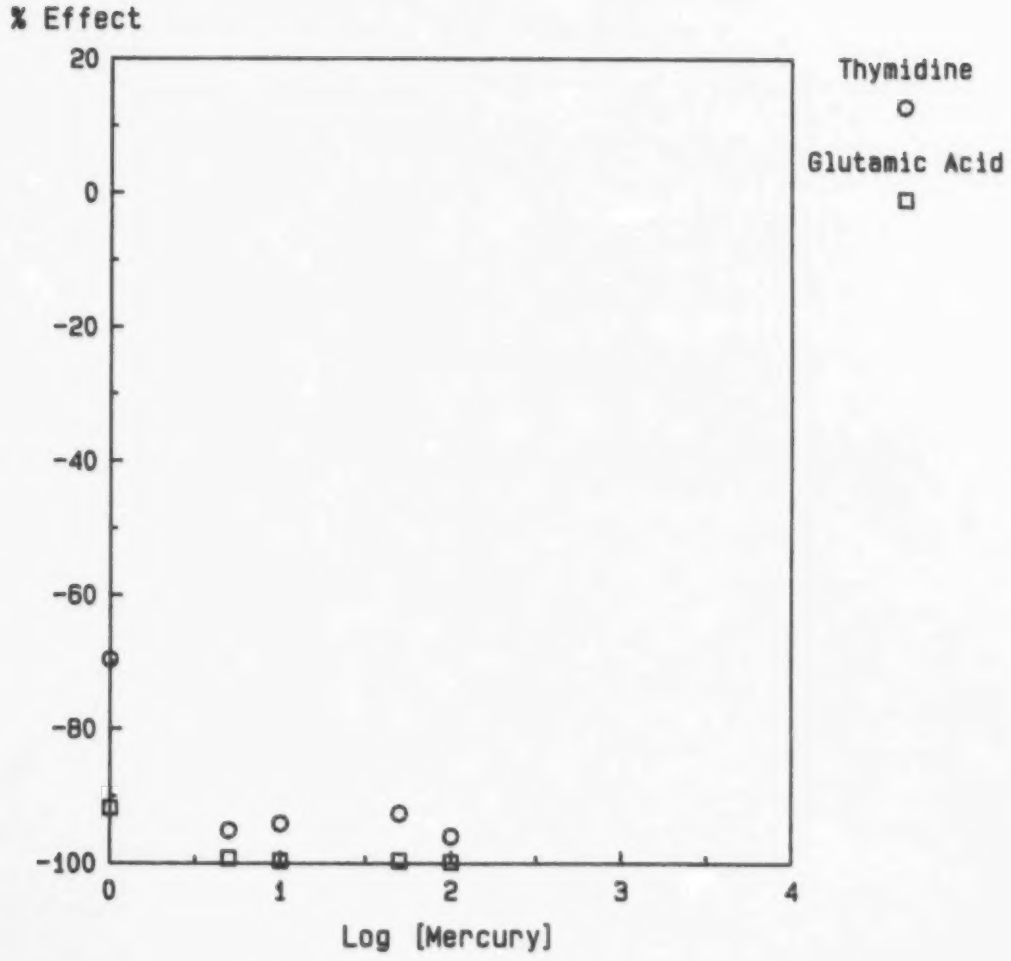

Figure 20: The effects of mercury on ^{14}C-glutamic acid and ^{3}H-thymidine uptake in slurries of sandy sediment.

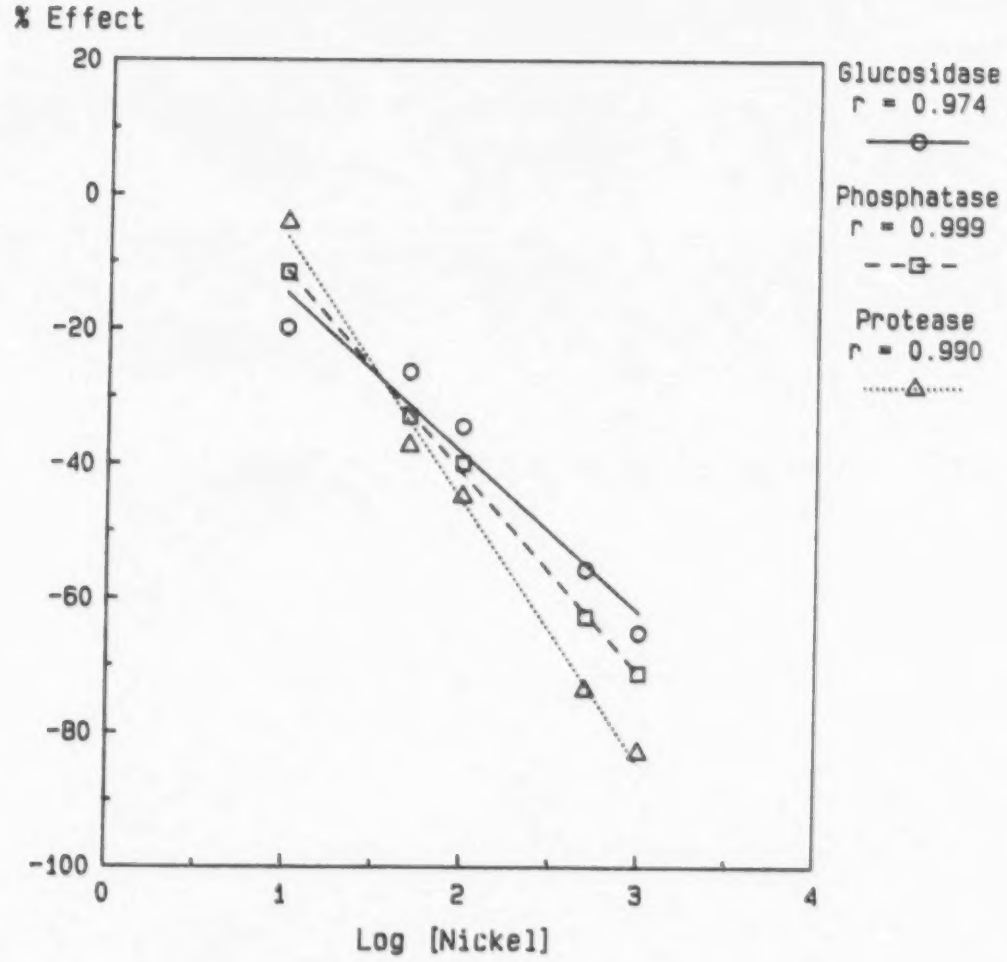

Figure 21: The effects of nickel on exoenzyme activity in slurries of sandy sediment.

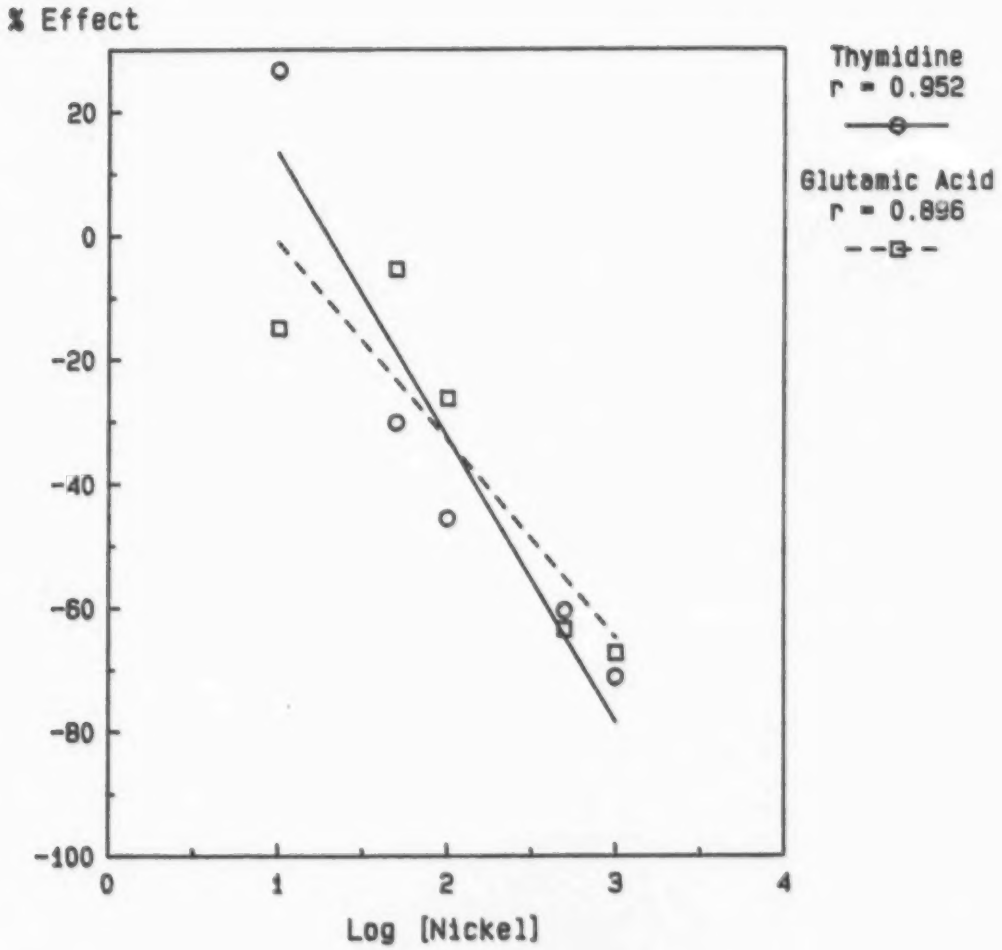

Figure 22: The effects of nickel on ^{14}C-glutamic acid and ^{3}H-thymidine uptake in slurries of sandy sediment.

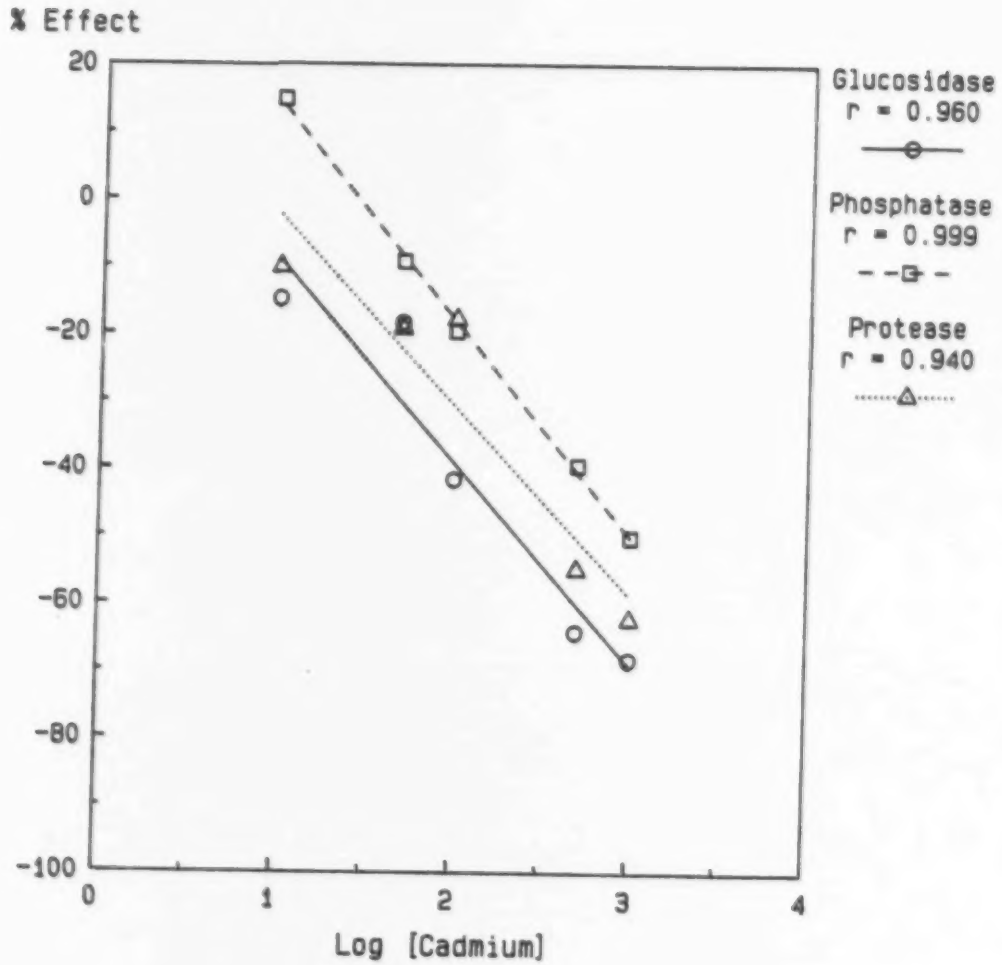

Figure 23: The effects of cadmium on exoenzyme activity in slurries of sandy sediment.

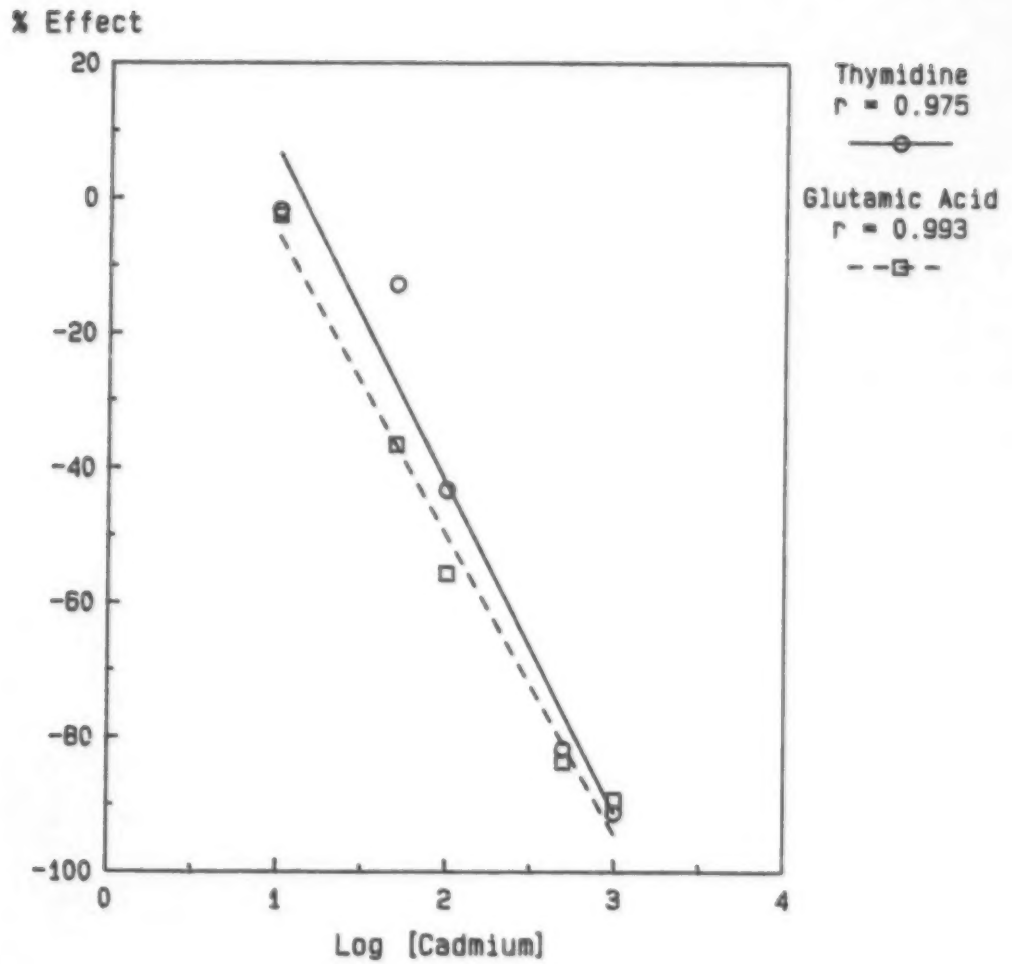

Figure 24: The effects of cadmium on ^{14}C-glutamic acid and ^{3}H-thymidine uptake in slurries of sandy sediment.

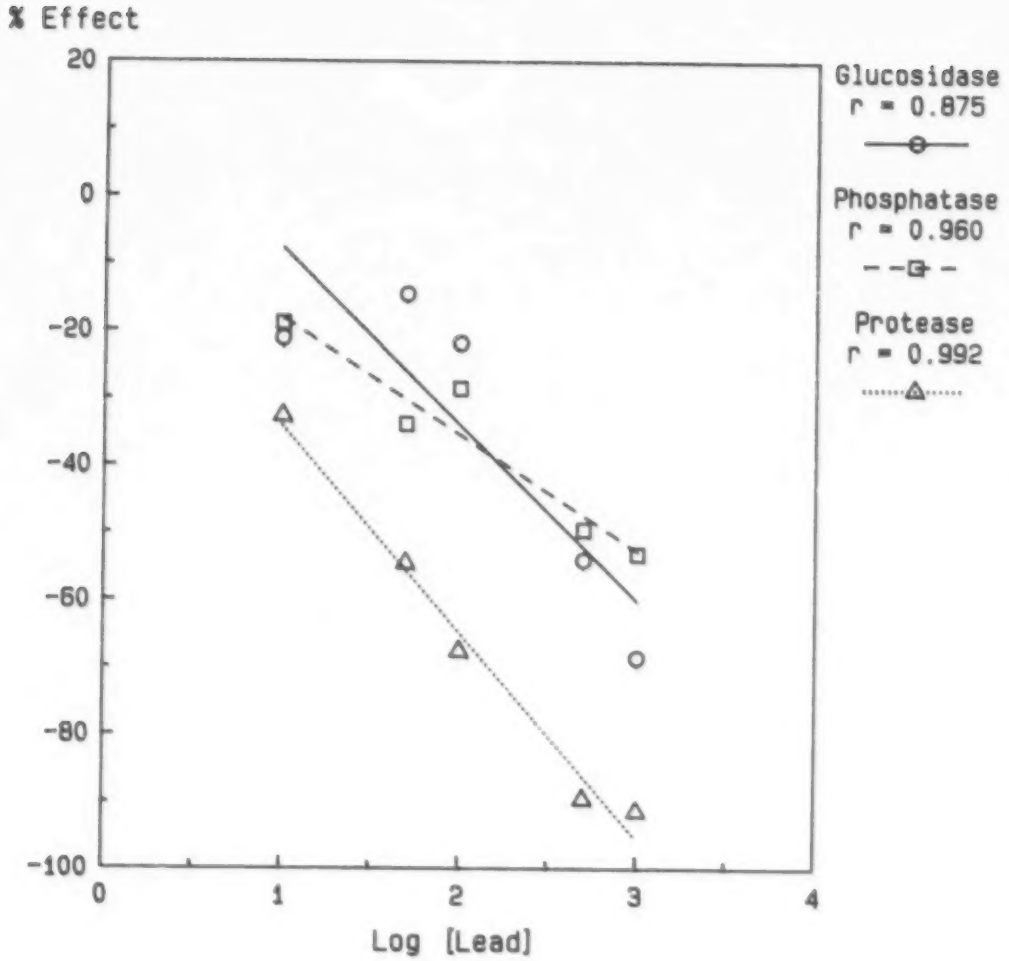

Figure 25: The effects of lead on exoenzyme activity in slurries of sandy sediment.

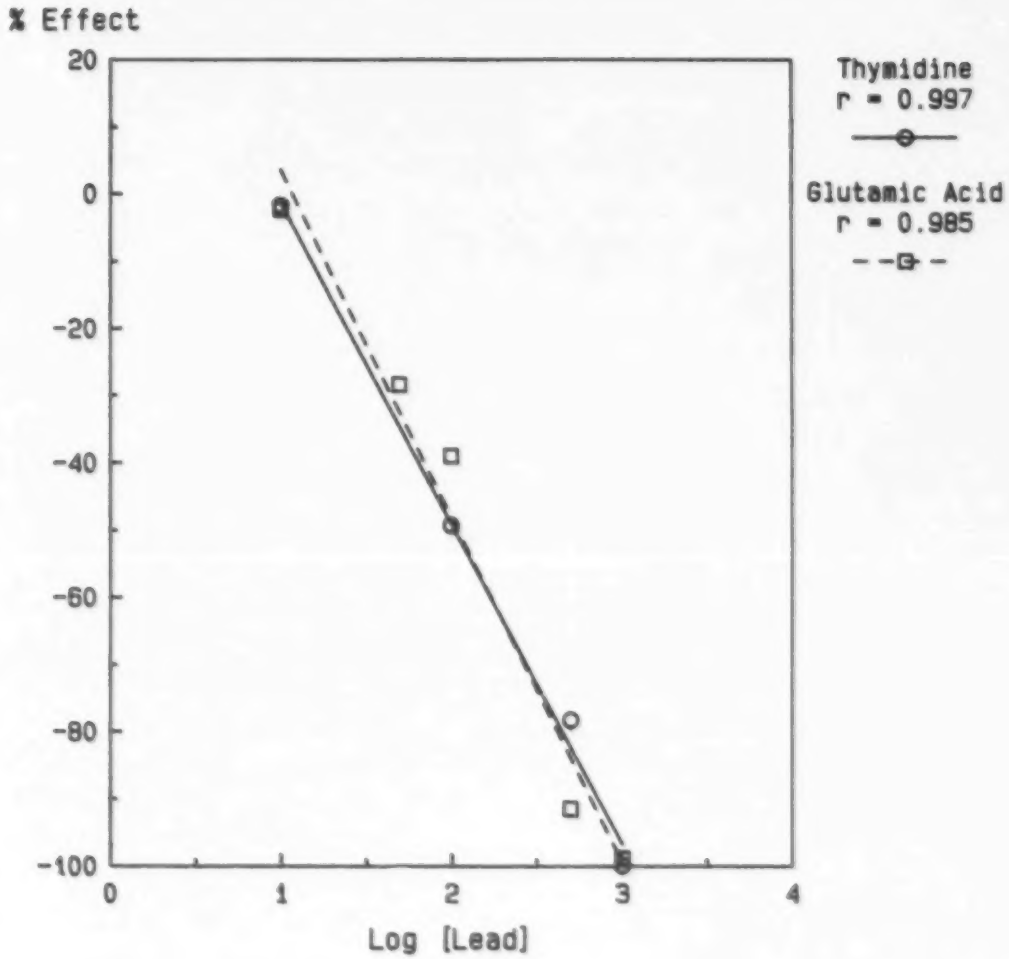

Figure 26: The effects of lead on ^{14}C-glutamic acid and ^{3}H-thymidine uptake in slurries of sandy sediment.

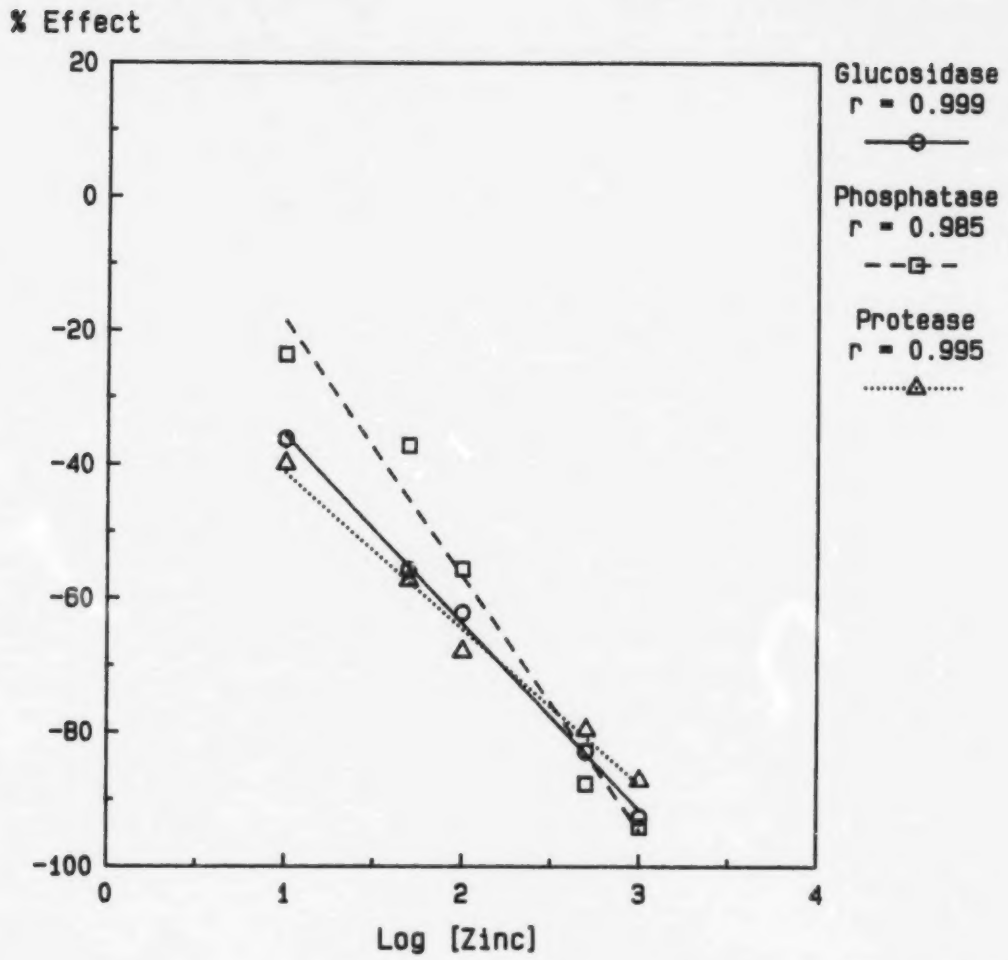

Figure 27: The effects of zinc on exoenzyme activity in slurries of sandy sediment.

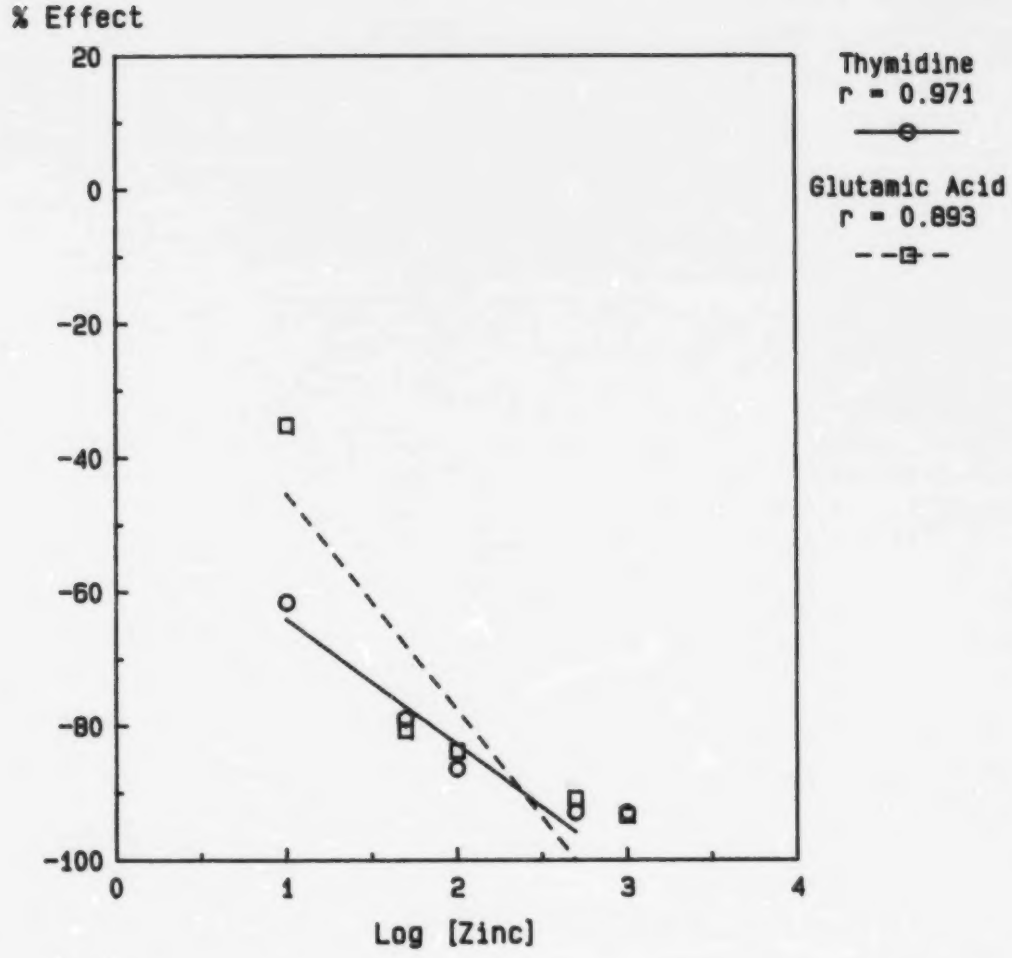

Figure 28: The effects of zinc on ^{14}C-glutamic acid and ^{3}H-thymidine uptake in slurries of sandy sediment.

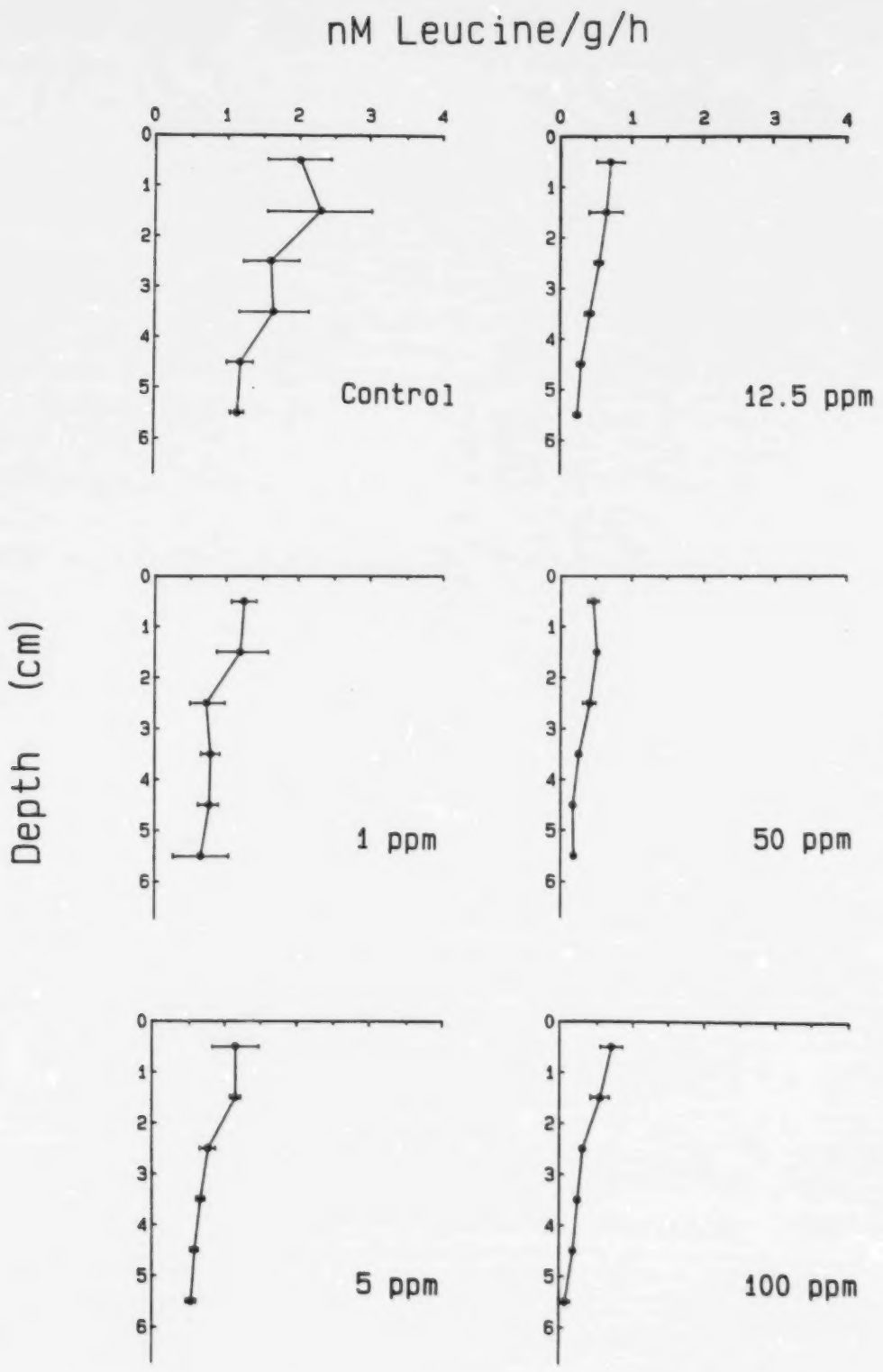

Figure 29: The effect of zinc on protease activity-depth profiles in cores of sandy sediment.

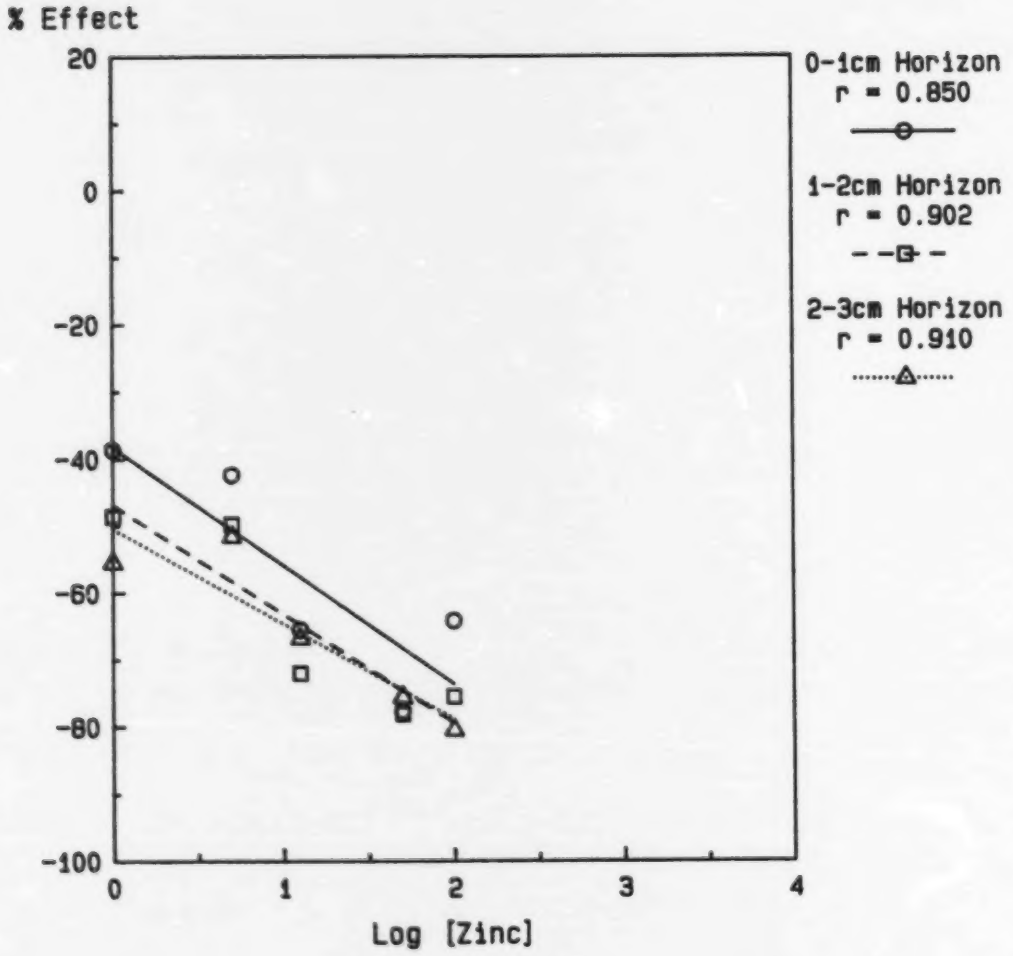

Figure 30: The effect of zinc on protease activity in sediment cores - 0-3cm.

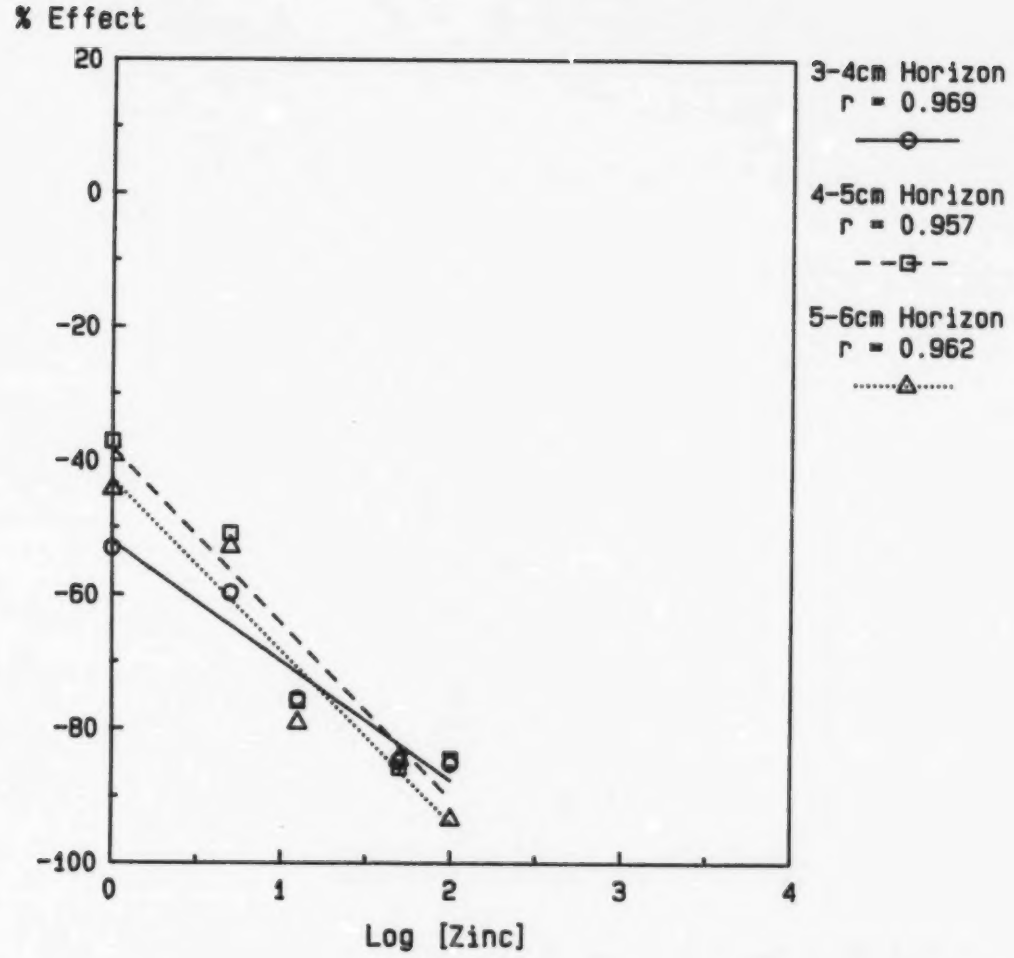

Figure 31: The effect of zinc on protease activity in sediment cores - 3-6cm.

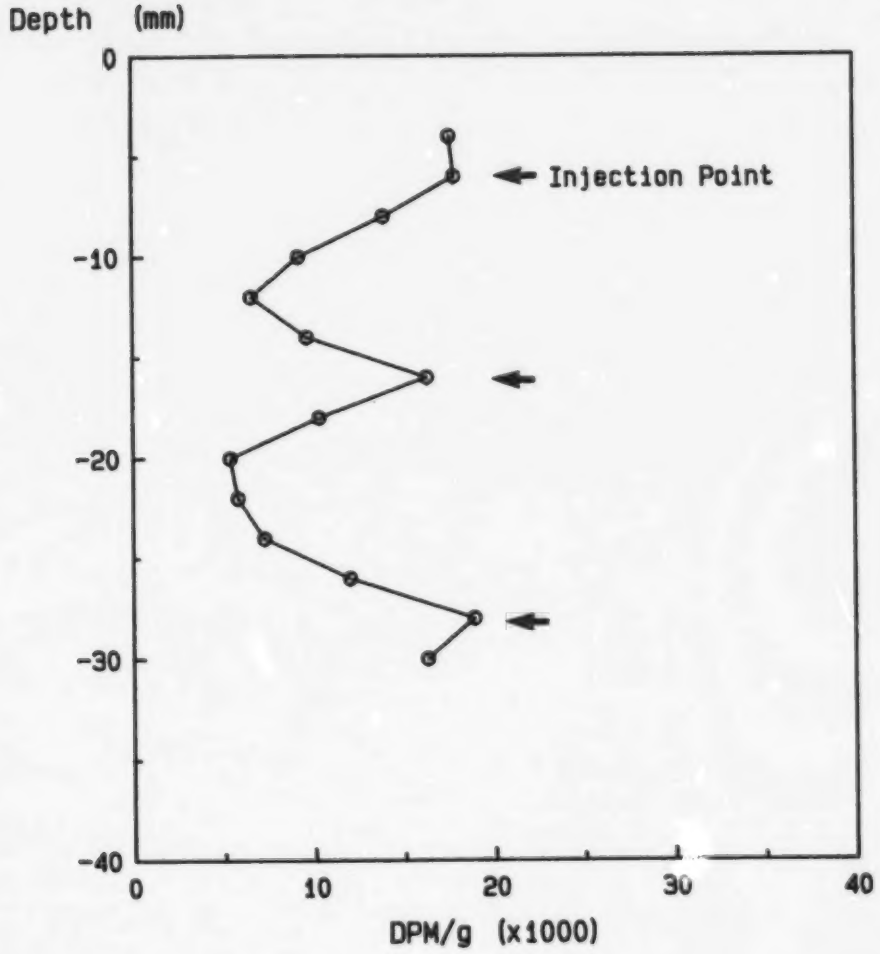

Figure 32: Vertical diffusion of ^{14}C-glutamic acid through a core of sandy sediment.

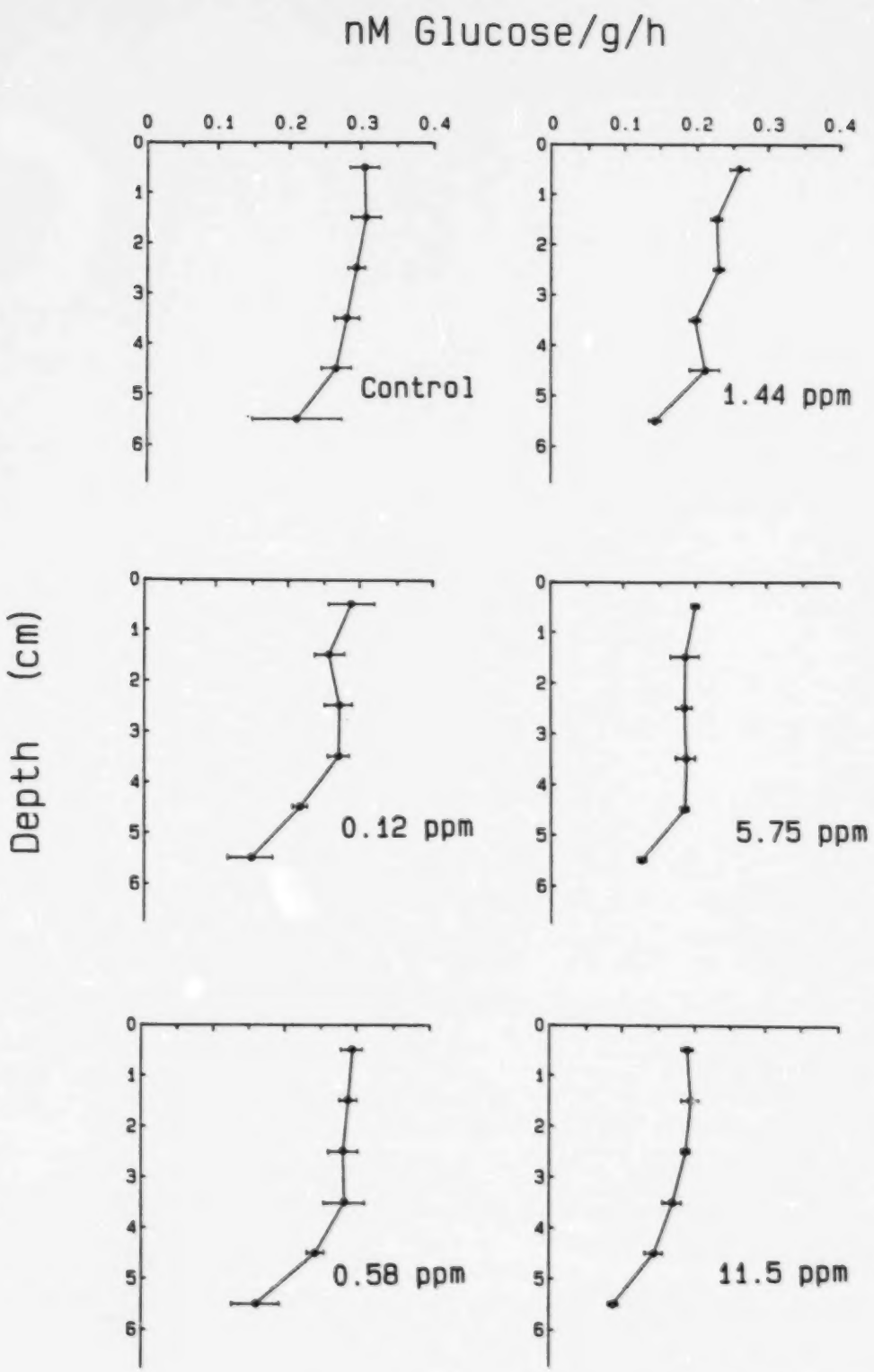

Figure 33: The effect of copper on the glucosidase activity - depth profiles in cores of sandy sediment.

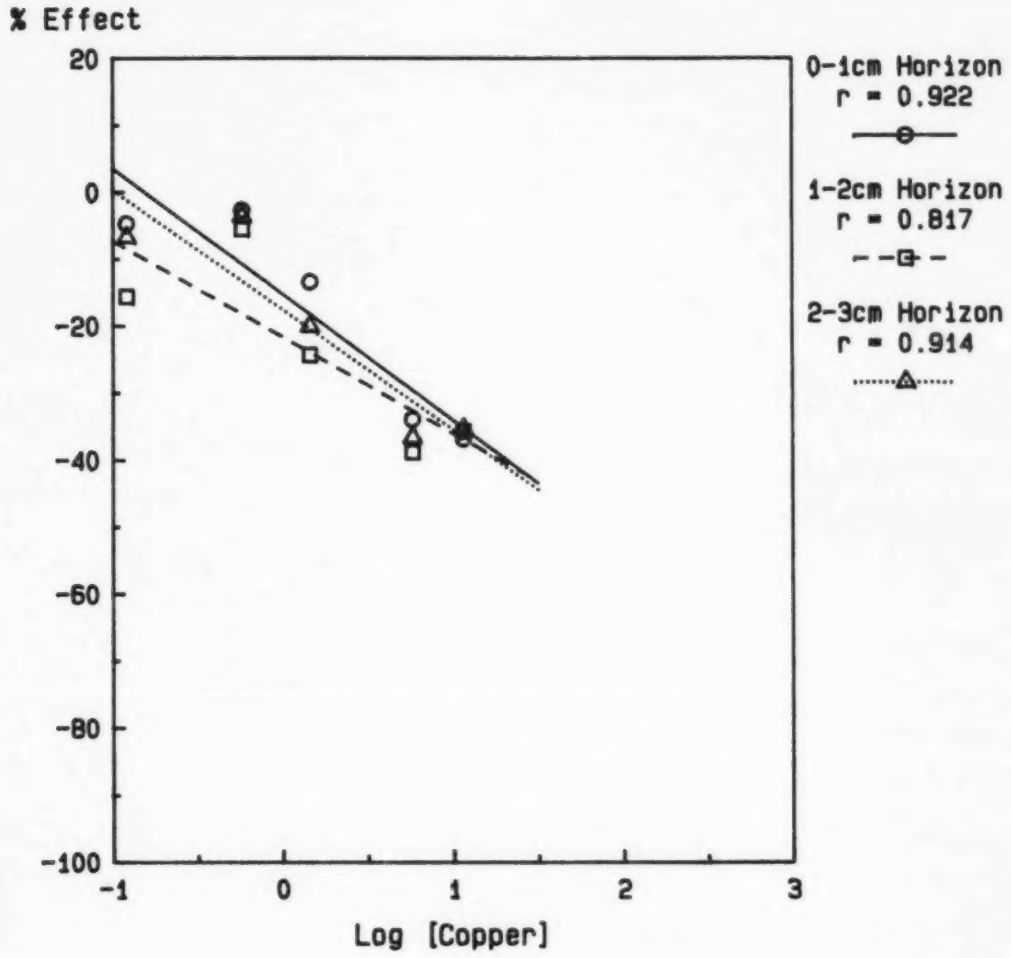

Figure 34: The effect of copper on glucosidase activity in sediment cores - 0-3cm.

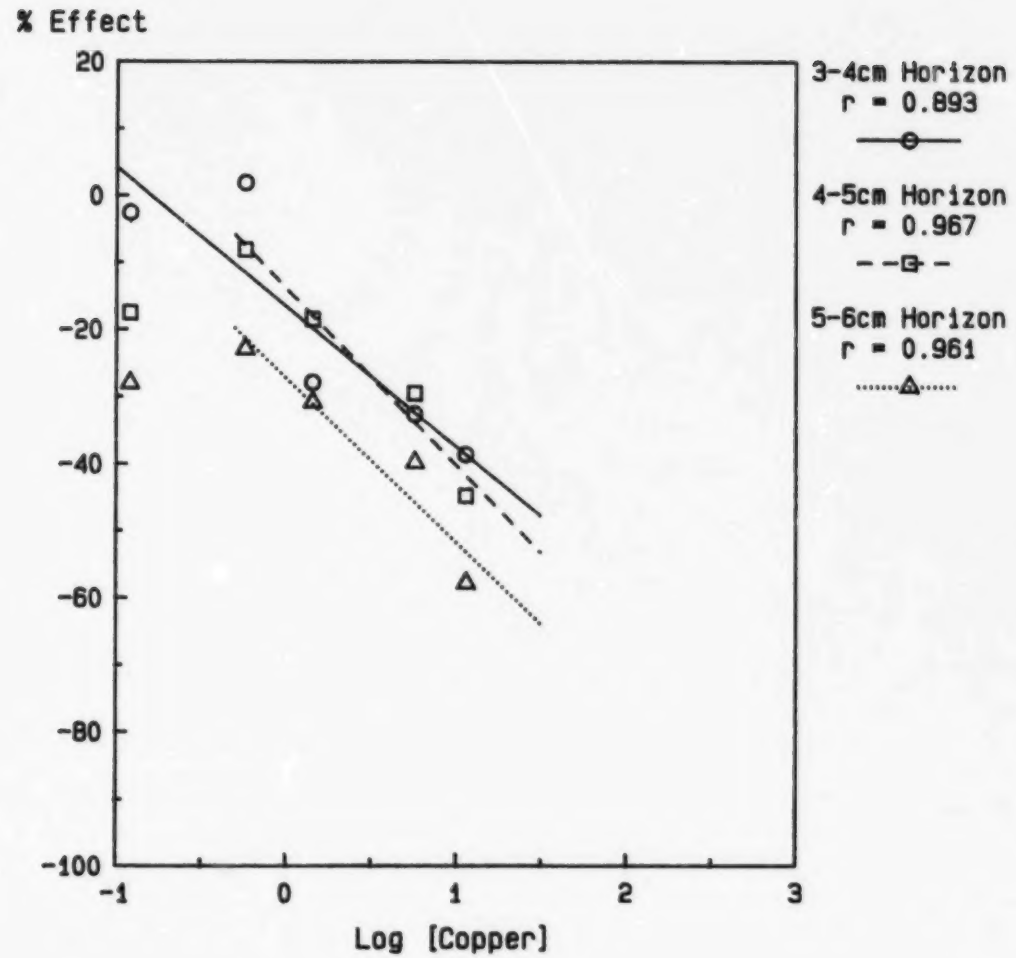

Figure 35: The effect of copper on glucosidase activity in sediment cores - 3-6cm.

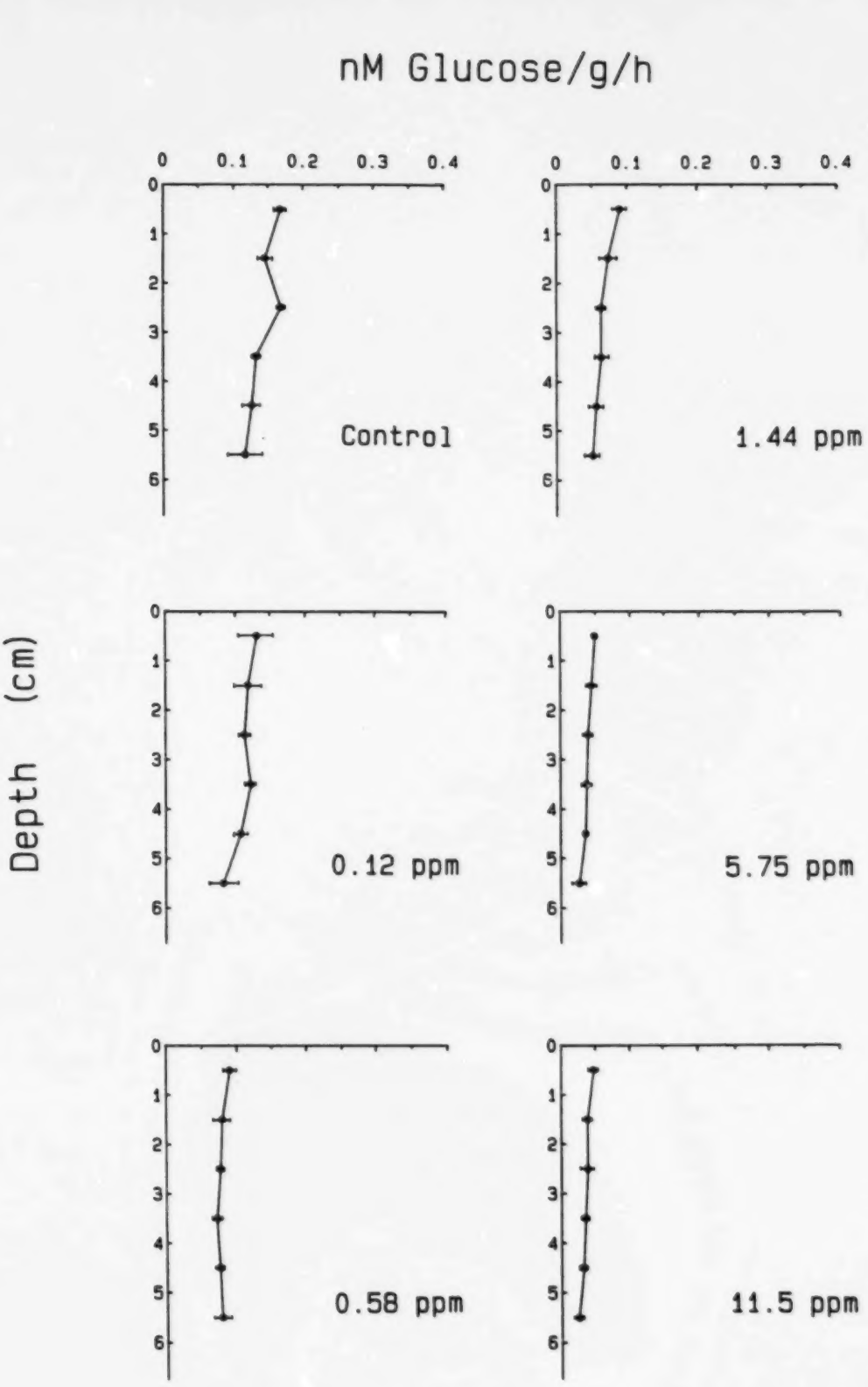

Figure 36: The effect of mercury on the glucosidase activity - depth profiles in cores of sandy sediment.

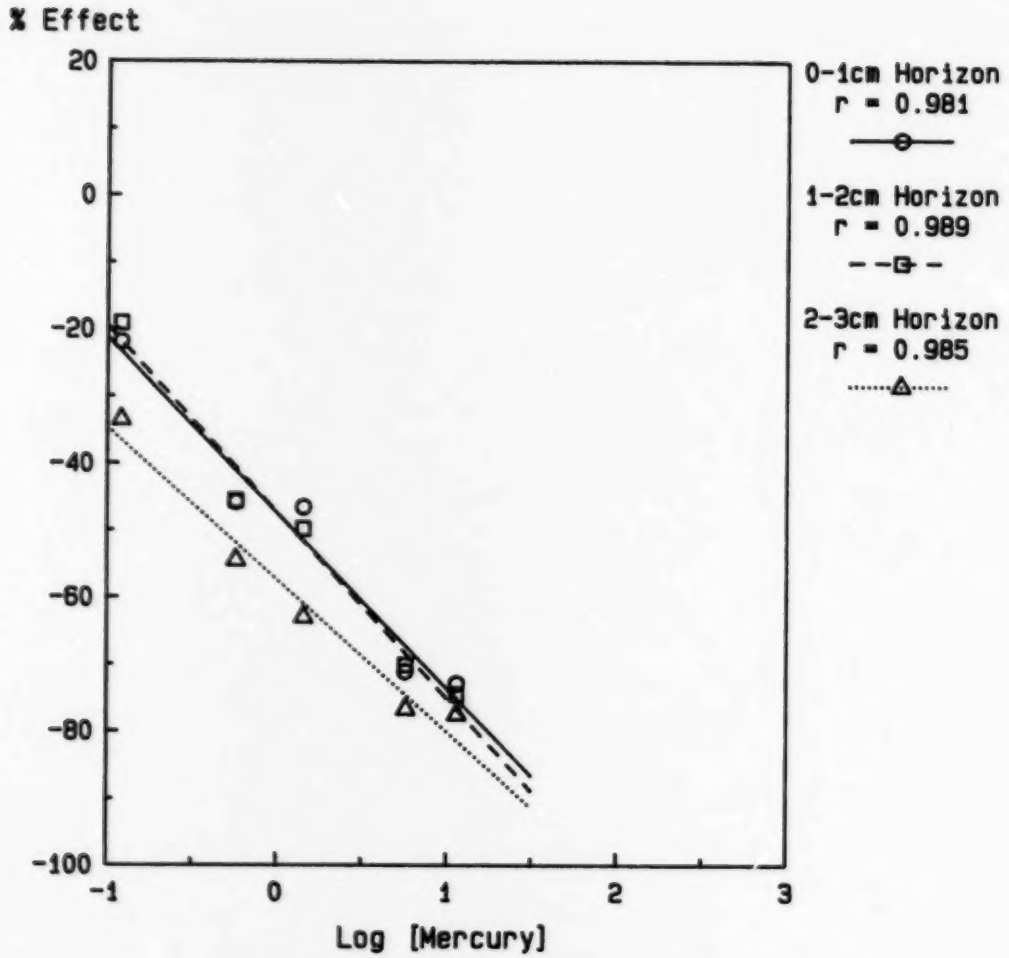

Figure 37: The effect of mercury on glucosidase activity in sediment cores - 0-3cm.

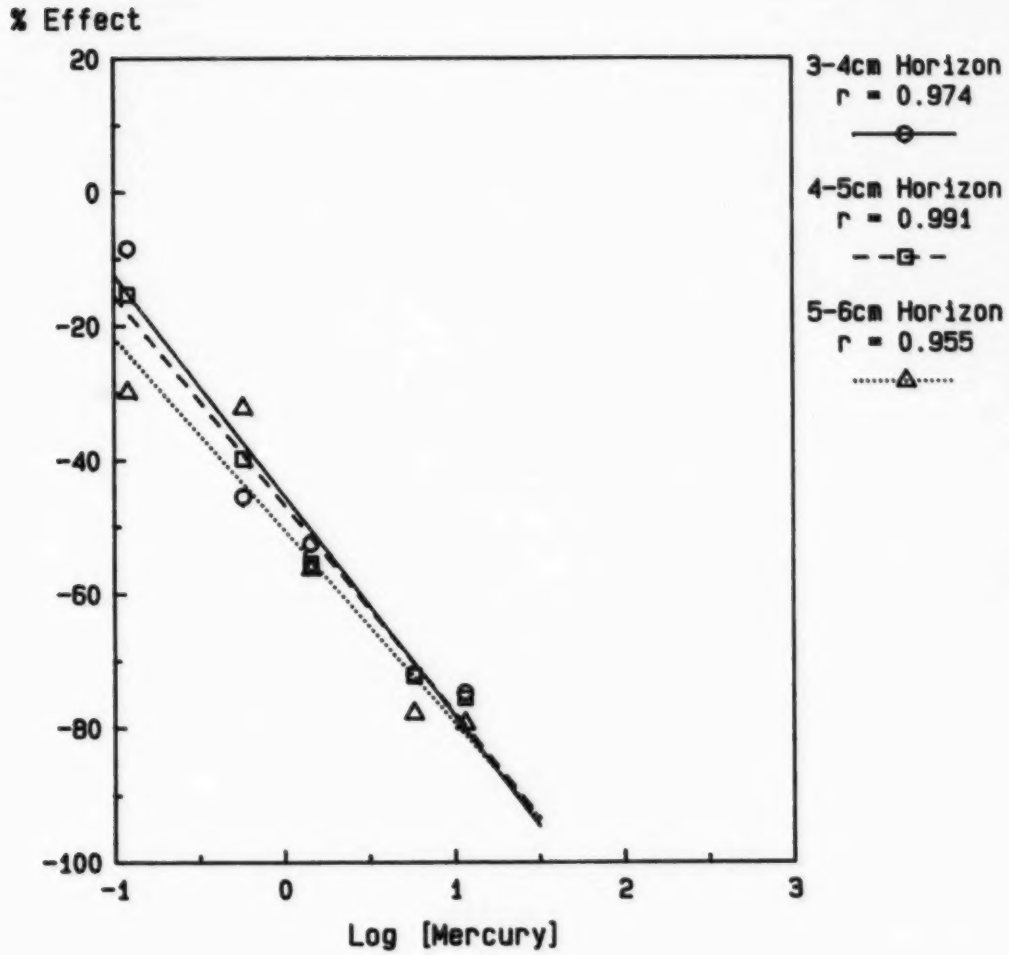

Figure 38: The effect of mercury on glucosidase activity in sediment cores - 3-6cm.

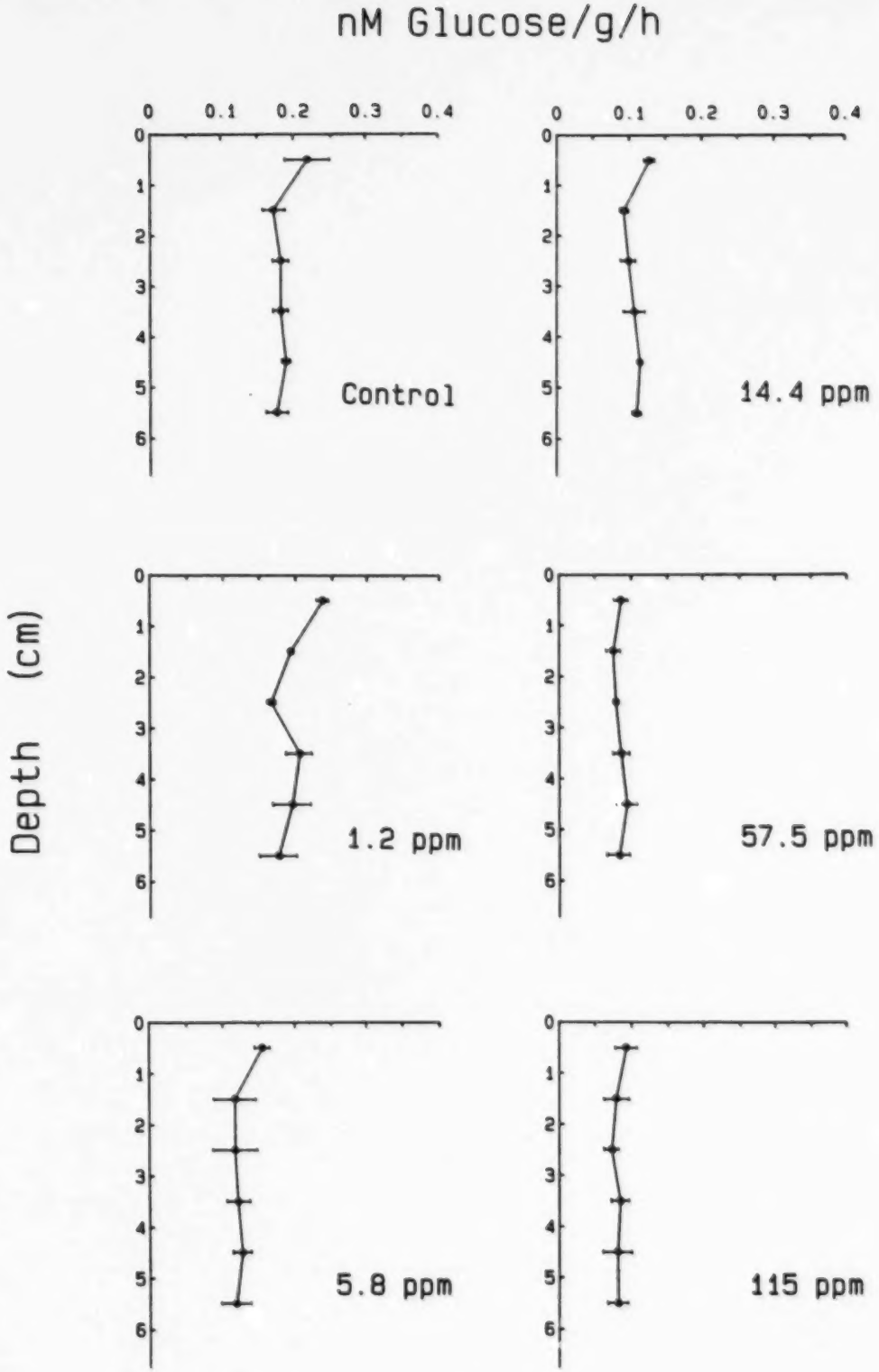

Figure 39: The effect of nickel on the glucosidase activity - depth profiles in cores of sandy sediment.

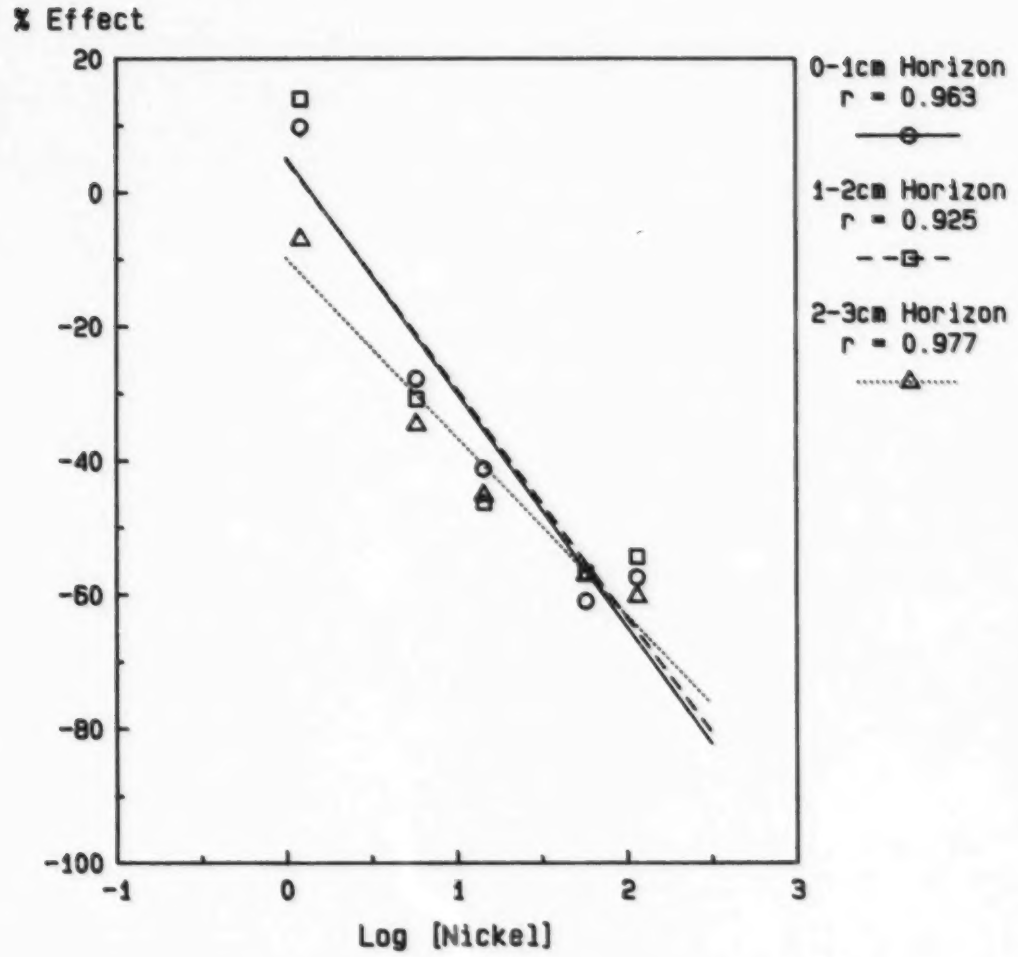

Figure 40: The effect of nickel on glucosidase activity in sediment cores - 0-3cm.

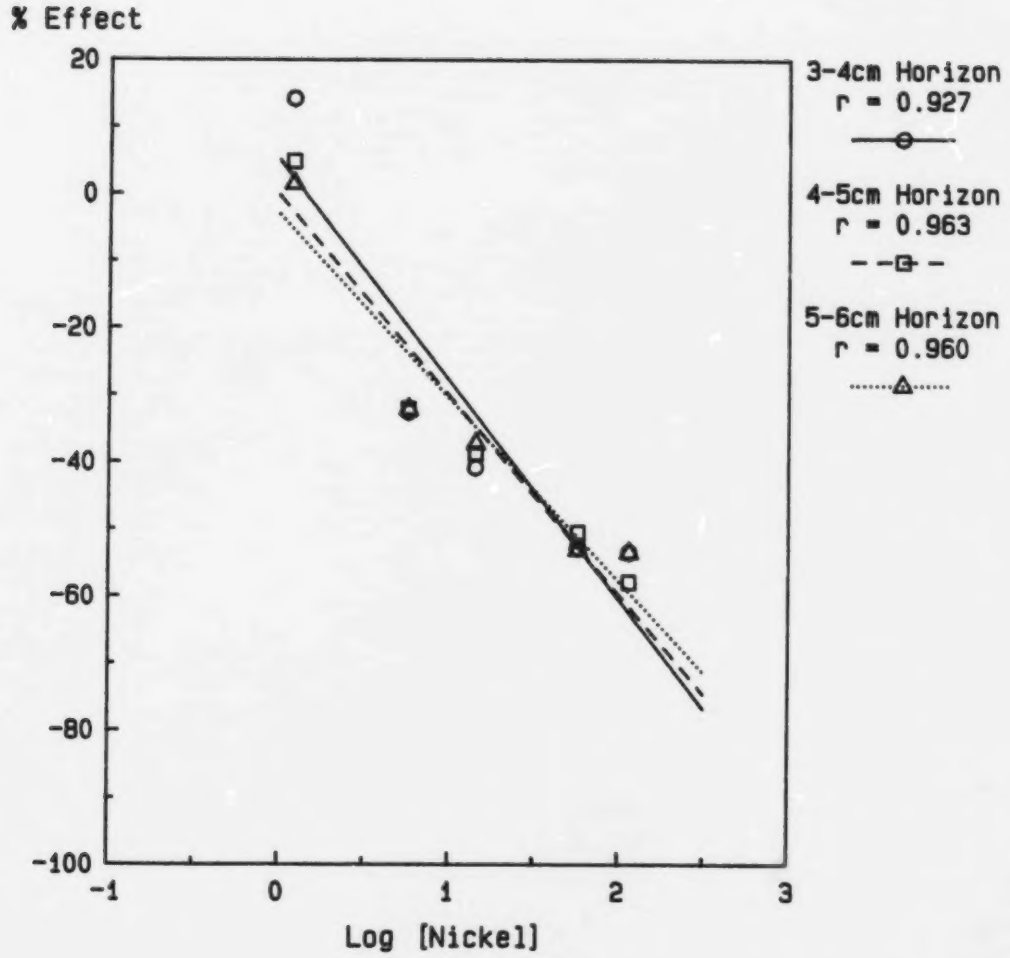

Figure 41: The effect of nickel on glucosidase activity in sediment cores - 3-6cm.

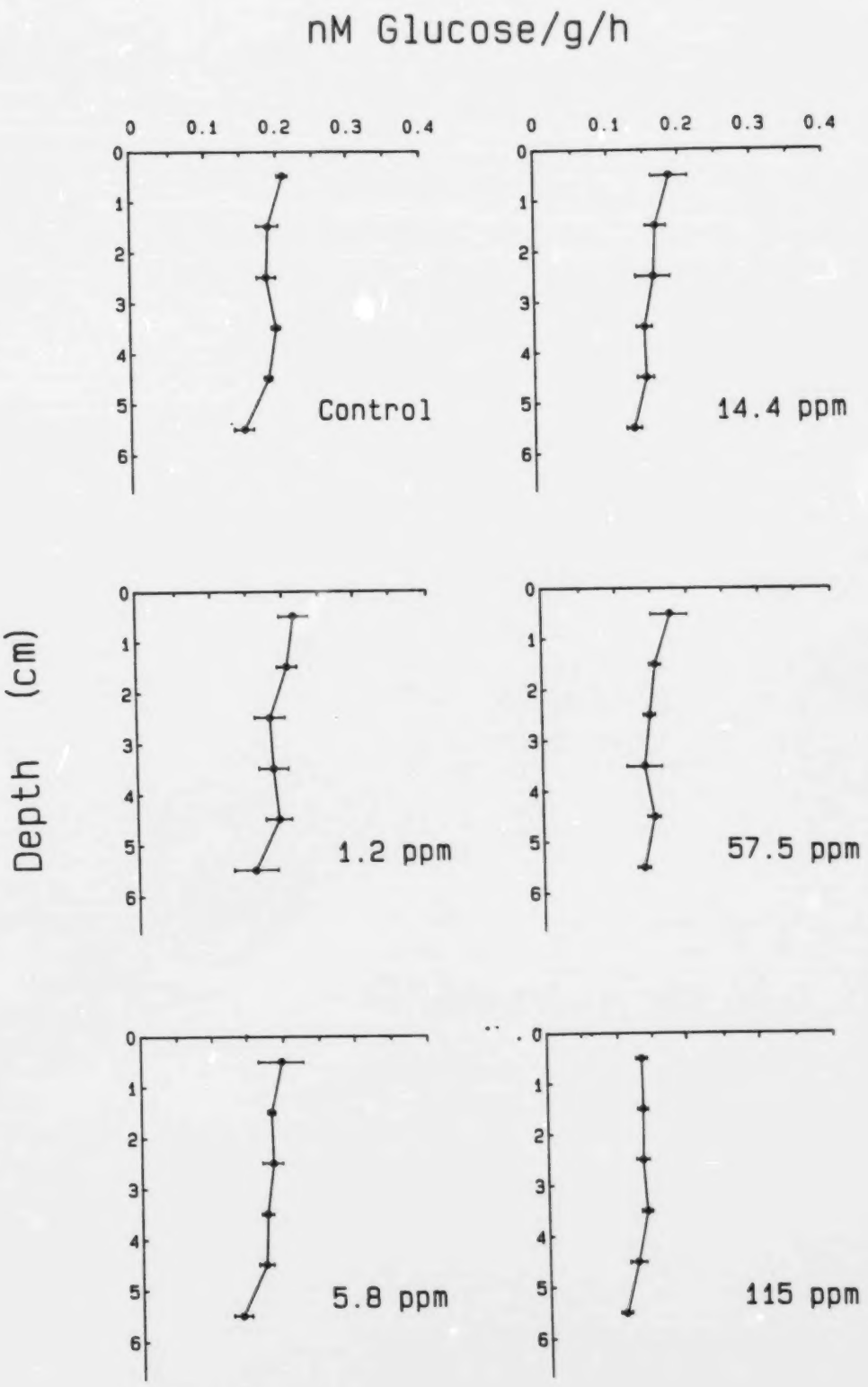

Figure 42: The effect of cadmium on the glucosidase activity - depth profiles in cores of sandy sediment.

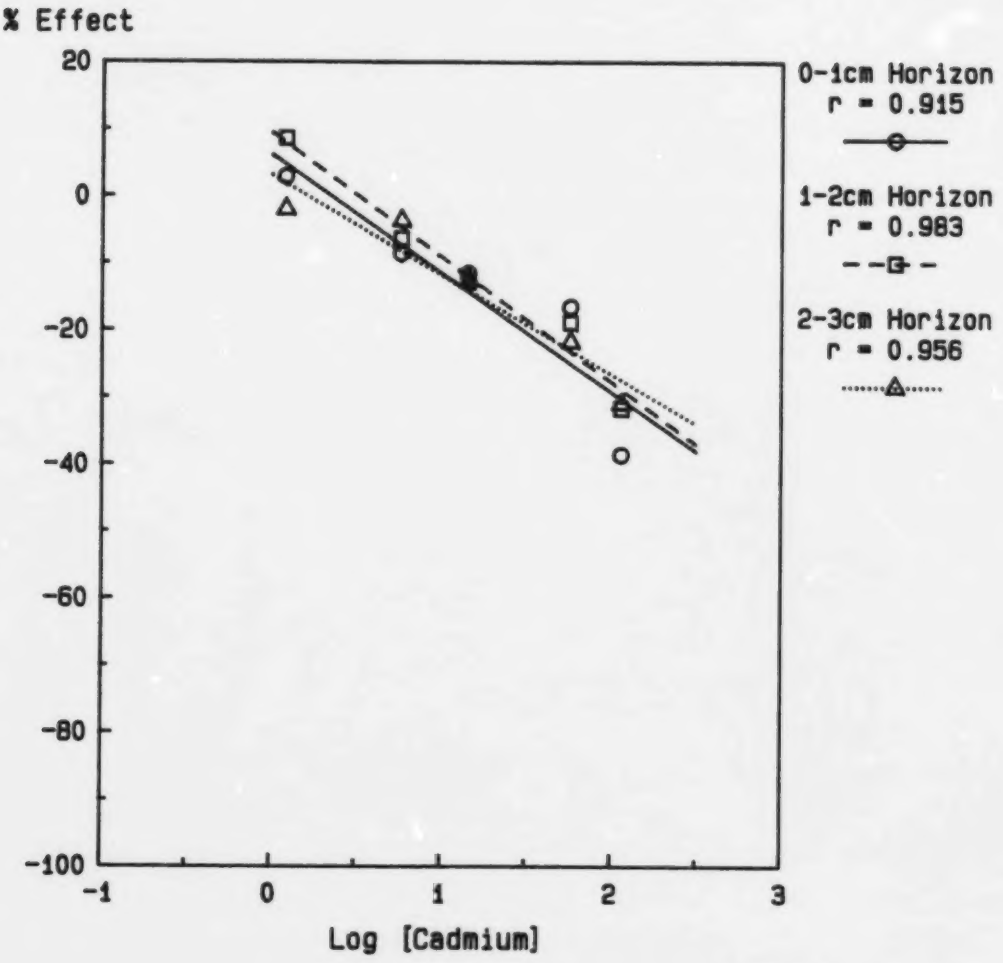

Figure 43: The effect of cadmium on glucosidase activity in sediment cores - 0-3cm.

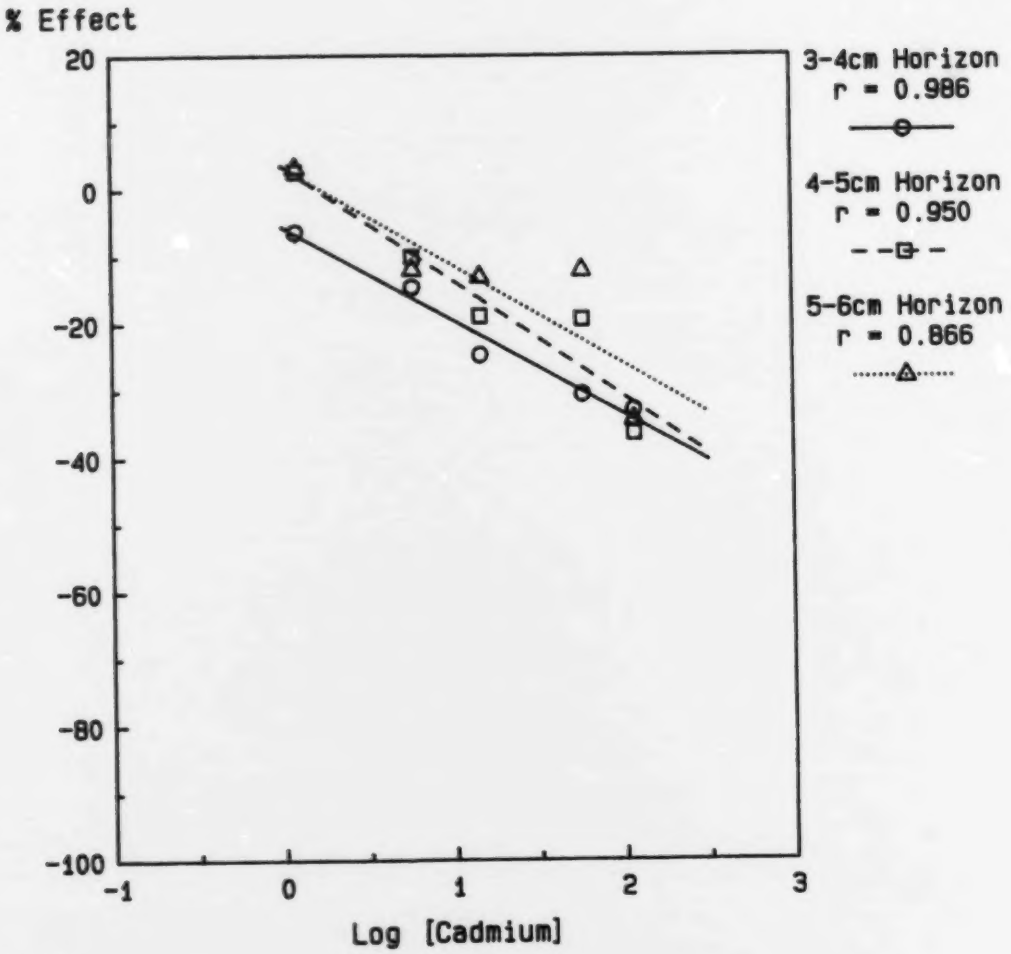

Figure 44: The effect of cadmium on glucosidase activity in sediment cores - 3-6cm.

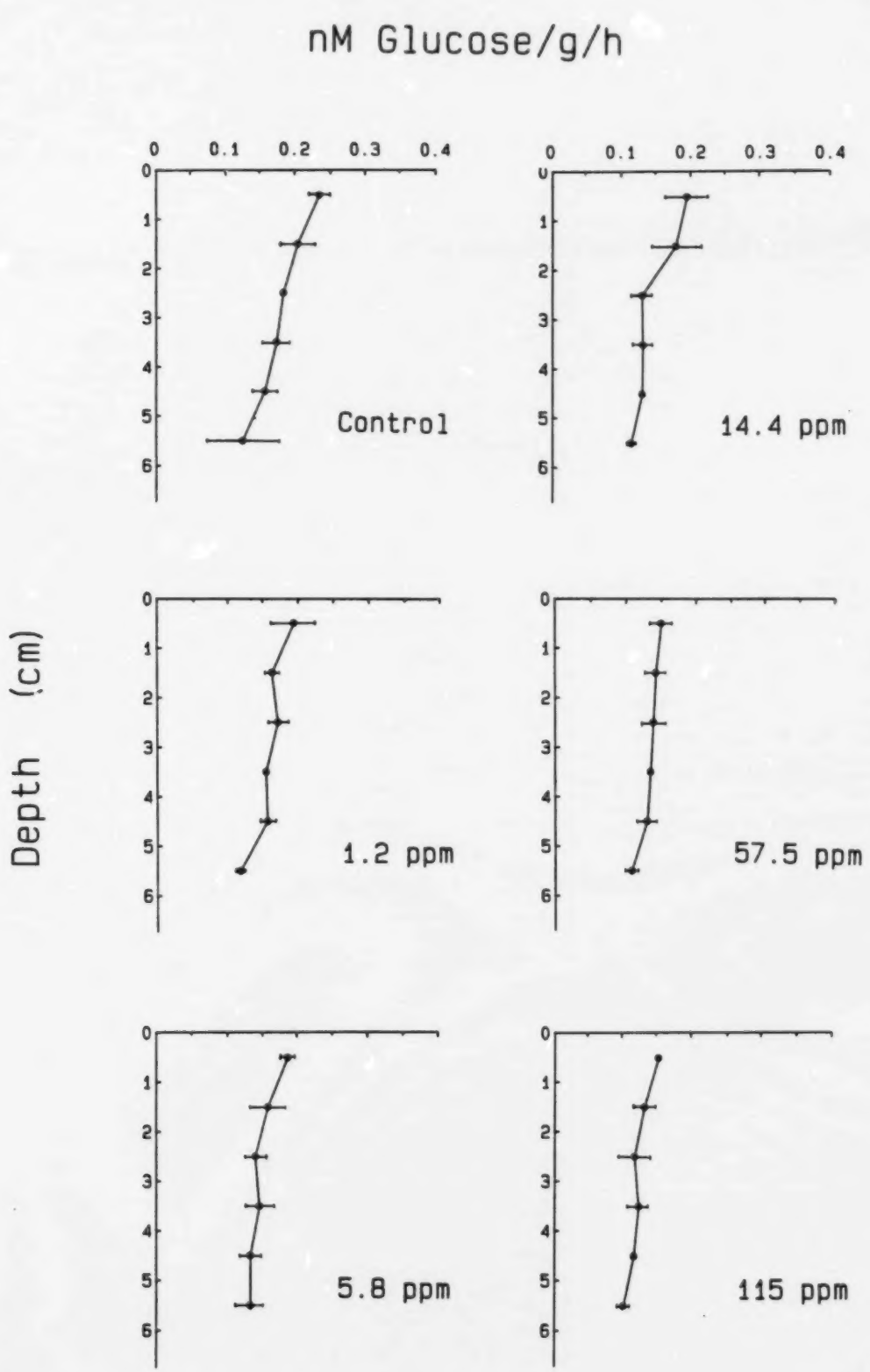

Figure 45: The effect of lead on the glucosidase activity - depth profiles in cores of sandy sediment.

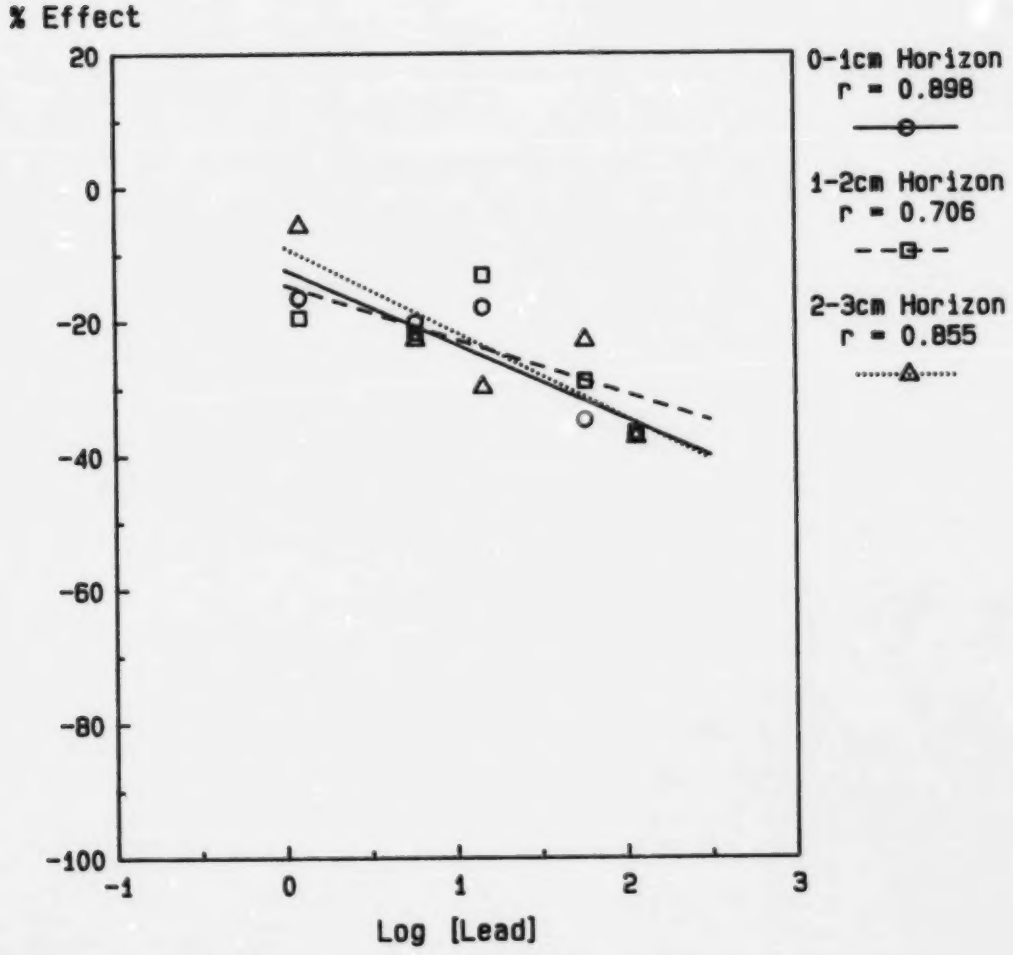

Figure 46: The effect of lead on glucosidase activity in sediment cores - 0-3cm.

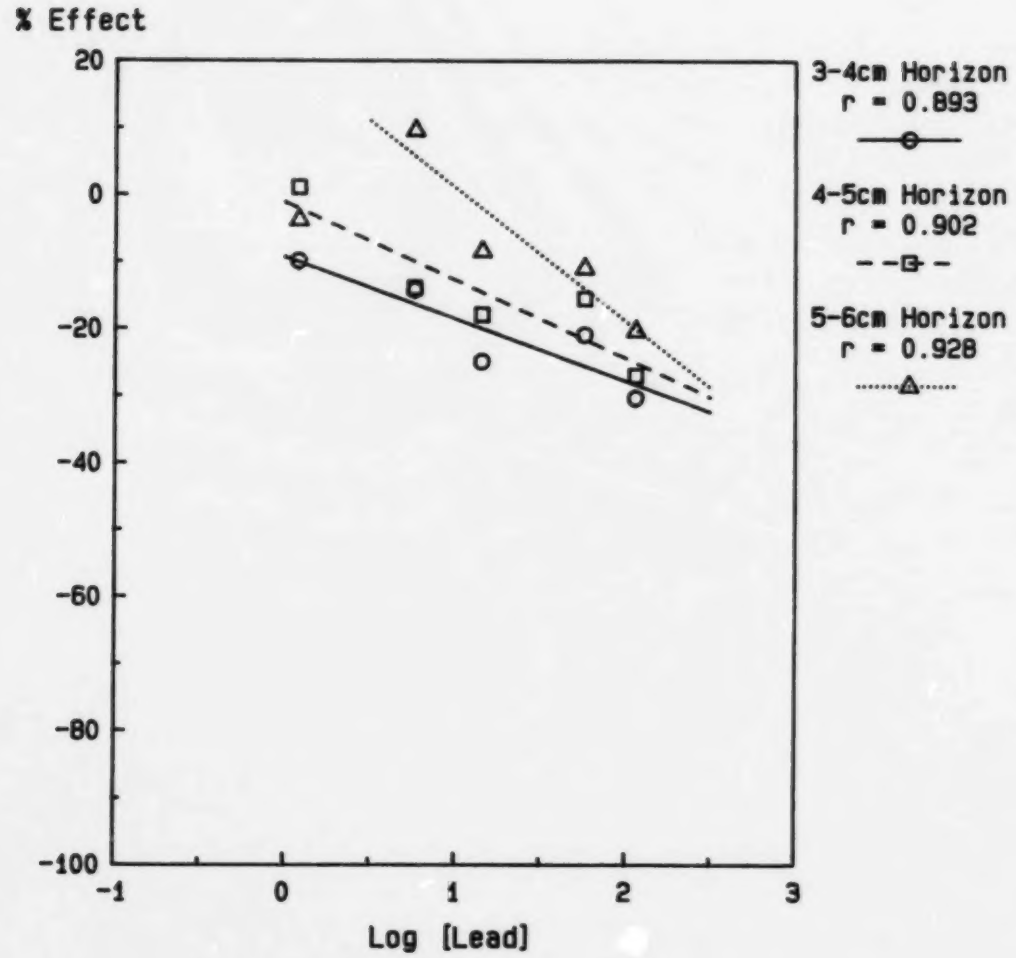

Figure 47: The effect of lead on glucosidase activity in sediment cores - 3-6cm.

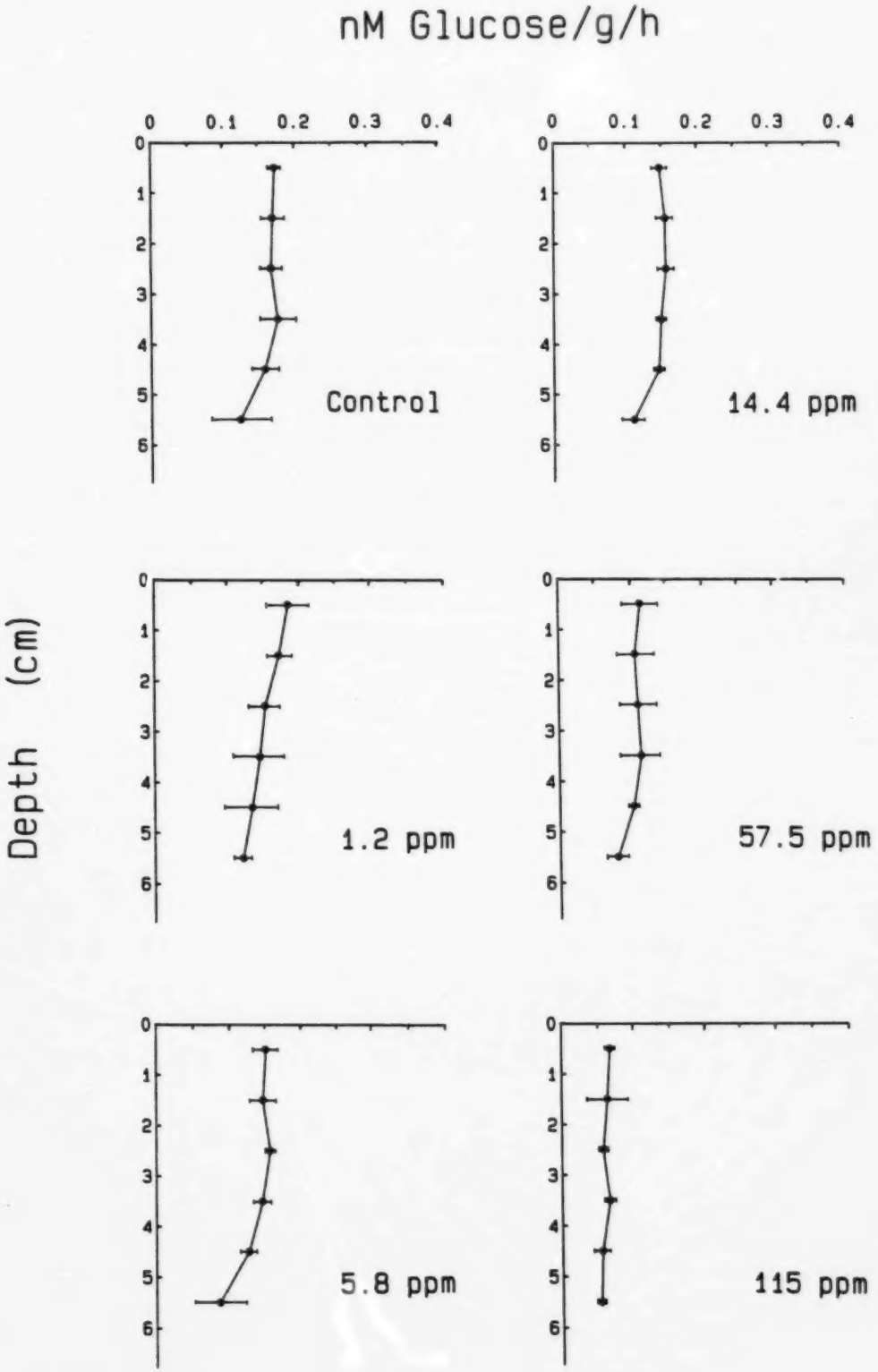

Figure 48: The effect of zinc on the glucosidase activity - depth profiles in cores of sandy sediment.

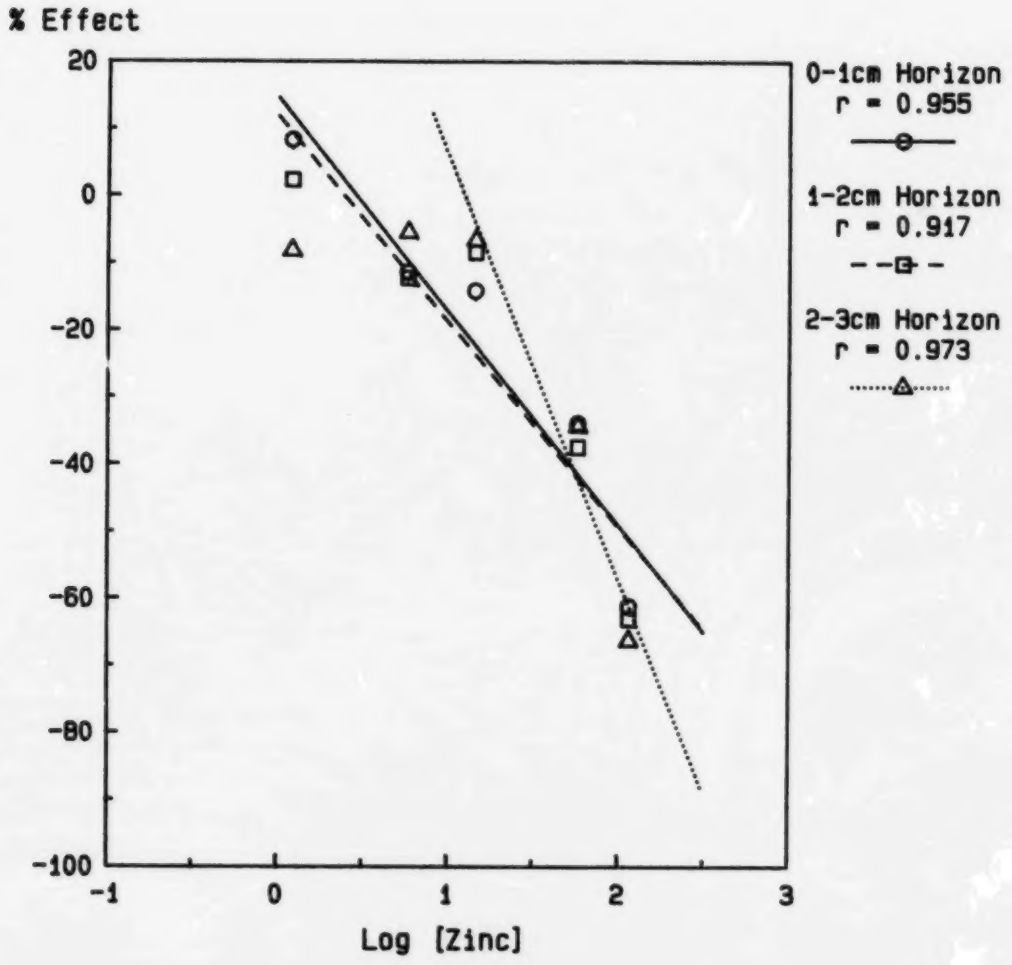

Figure 49: The effect of zinc on glucosidase activity in sediment cores - 0-3cm.

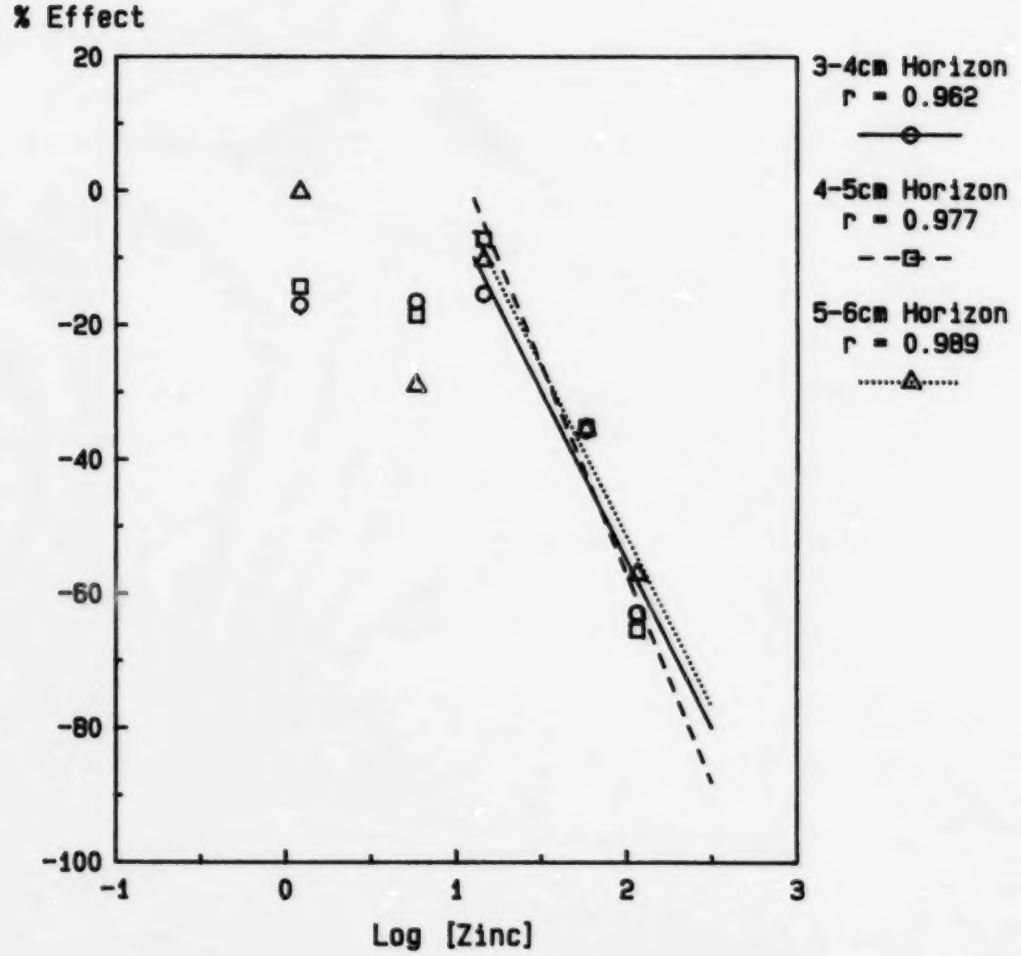

Figure 50: The effect of zinc on glucosidase activity in sediment cores - 3-6cm.

12.0 APPENDICES

APPENDIX A: Method for Exoenzyme Activity Bioassay in Sediment Slurries

Introduction: Since bacteria are unable to assimilate large macromolecules directly, extracellular enzymatic breakdown must occur before uptake is possible. This breakdown is accomplished by exoenzymes induced by the presence of a suitable substrate. The activity of specific inducible exoenzymes can be measured through the use of substrate analogues conjugated with the highly fluorescent compound, methylumbelliferone (MUF) (Meyer-Reil, 1986).

In the following method, the enzyme specific substrates 4-methylumbelliferyl-B-D-glucoside (for glucosidase), 4-methylumbelliferyl-phosphate (for phosphatase) and L-leucine-4-methylcoumarinyl-amide (for protease) are used to quantify exoenzymatic activity in slurries of sediment suspended in sea water. Enzymatic hydrolysis of these MUF-substrates yields equimolar amounts of methylumbelliferone and product (eg. glucose in the case of MUF-glucoside) (Fig. 51). The high flourescence of methylumbelliferone allows measurement of enzymatic activity at ambient temperatures, at substrate concentrations that closely resemble natural background concentrations with short incubation times. This allows the rapid measurement of a physiological response by bacteria to the toxicity of a compound, under conditions similar to those found in nature.

Outline of Method: A toxicant is added to a mixture of sediment suspended in synthetic sea water (1:15 by volume) and incubated in the presence of a 5 uM MUF-substrate. Following incubation, the slurry is frozen to arrest metabolic activity. For analysis, the slurry is centrifuged, after thawing in an ice bath. The supernatant is decanted into a cuvette and a buffer is added to maximize the fluorescence of the liberated MUF (Fig. 52). Enzyme activity is calculated via quantification of the amount of MUF liberated over the incubation period. Compound toxicity is calculated as the EC_{50}, the concentration of toxicant that results in a 50% decrease in enzyme activity.

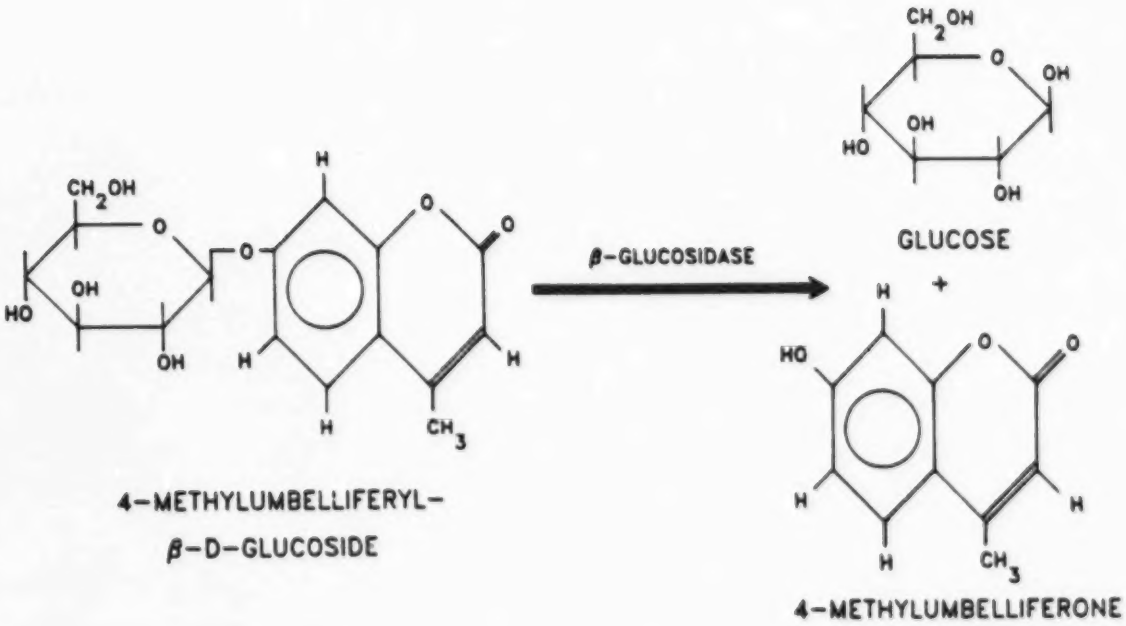

Figure 51: Action of B-Glucosidase on Methylumbelliferyl substrate analogue, 4-Methylumbelliferyl-B-D-Glucoside.

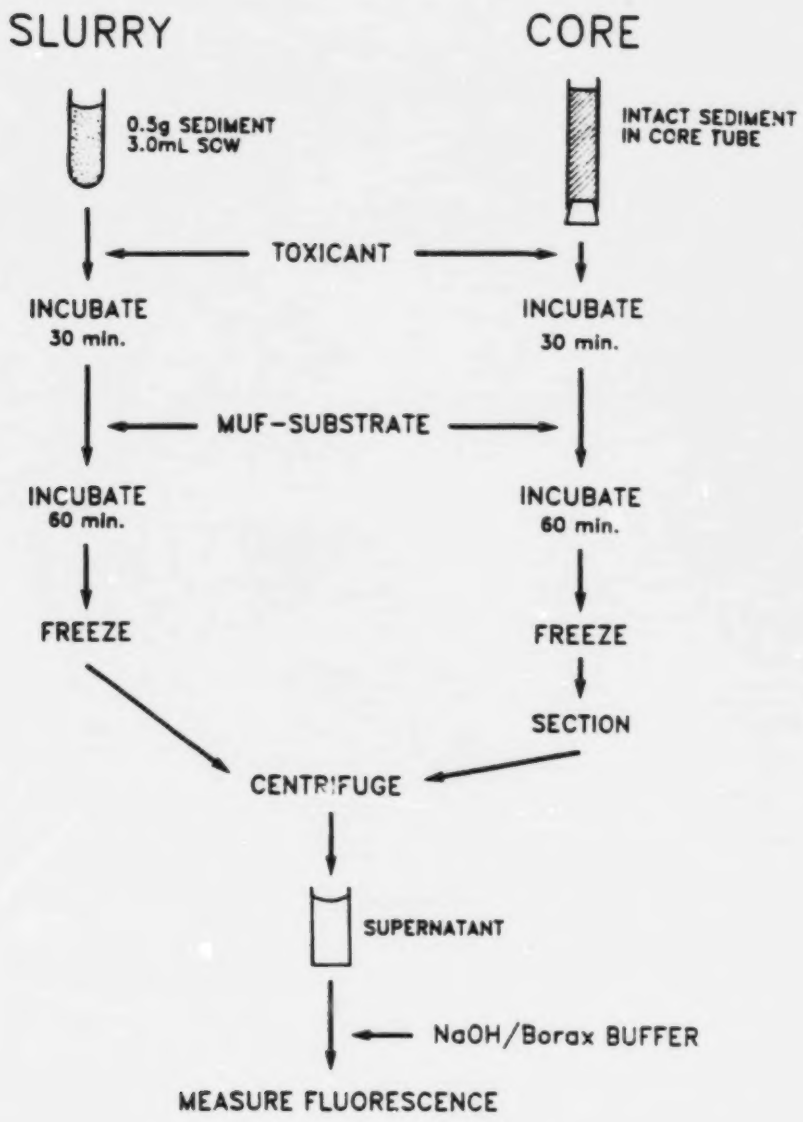

Figure 52: Outline of Enzyme Bioassay Protocol.

Reagents: **Synthetic Ocean Water (SOW)** is prepared according to Morel et al. (1979). Reagent grade salts are added, in the following order, to 1 L of distilled water:

Salt	Amount (g)
NaCl	24.53
$CaCl_2.2H_2O$	1.54
KBr	0.10
NaF	0.003
KCl	0.70
H_3BO_3	0.03
Na_2SO_4	4.09
$NaHCO_3$	0.20
$SrCl_2.6H_2O$	0.017
$MgCl_2.6H_2O$	11.10

The mixture is adjusted to pH 8.0 using 1N $NaHCO_3$.

Metal stock solutions used as reference toxicants in the present study are prepared in SOW as follows:

Salt	Stock Concentration (M)
$HgCl_2$	0.0015
$CuCl_2.2H_2O$	0.0047
$CdSO_4$	0.0267
$ZnSO_4.7H_2O$	0.0459
$PbCl_2$*	0.0145
$NiCL_2.6H_2O$	0.0511

* Prepared in distilled water to prevent precipitation.

Methylumbelliferyl substrate stocks are prepared using 4-Methylumbelliferyl-β-D-glucoside, 4-Methylumbelliferyl-phosphate (Sigma Chemical Co.) and L-Leucine-4-methyl coumarinyl-7-amide HCl (Fluka Chemical Corp.), at a concentration of 5 mM in 10% methanol in SOW. MUF substrates are stored at -20°C when not in use. For use in slurries, working stock solutions are prepared by making 1:33 dilutions in SOW.

NaOH/Borax Buffer (pH 10.8) is prepared with 0.2 M NaOH and 0.05 M sodium tetraborate. The buffer consists of 50 mL of sodium tetraborate solution and 67 mL of NaOH and adjusted to a final volume of 200 mL with distilled water.

Methylumbelliferone Standard solution (2.5 mM) was prepared using methylumbelliferone (Sigma Chemical Co.) in reagent grade methanol. A working solution was prepared by diluting 200 uL of the stock in 200 mL of SOW. The final standard solution was prepared with 1.2 mL of working solution and 1.8 mL SOW. This yielded the required 3.0 mL assay volume at a concentration of 1 uM.

Prior to use, all glassware used in the experiment was soaked overnight in 5% HNO_3 and rinsed at least 5 times with distilled water.

Sediment Preparation: Following field collection, sediment cores are stored in running sea water tanks. Immediately prior to analysis, the 0-2 cm layer of the sediment cores is removed, thoroughly mixed in a beaker with a glass stirring rod and maintained under ambient temperature conditions.

Assay Procedure:

1) Preweighed 10mL test tubes are prepared in triplicate as follows for all metals except lead:

Metal Sol'n (uL)	SOW (mL)
0	3.0
10	2.99
50	2.95
100	2.9
500	2.5
1000	2.0

For lead, 2.0 mL of SOW is added to each tube and 1.0 mL of distilled water less the volume of metal stock is added. When dispensed as above, these stock solutions yield concentrations of 1, 5, 10, 50 and 100 ppm of Hg and Cu and 10, 50, 100, 500, and 1000 ppm of Cd, Zn, Pb and Ni.

2) Homogeneously mixed sediment is added to each tube (0.2 cm^3).

3) Each tube is then agitated for about 5 sec using a vortex mixer and incubated for 30 min at ambient temperature.

4) After preincubation, 100 uL of the working MUF-substrate solution is added to each tube (final substrate concentration of 5 uM) and the time recorded prior to reincubation, with agitation every 20 min.

5) After 60 min (30 min for phosphatase), the tubes are placed in dry ice to arrest all enzymatic activity. Once frozen, the tubes can be stored below 0°C until activity measurement by fluorescence.

6) Experimental blanks (SOW with sediment) and a 1.0 uM MUF standard with sediment are prepared concurrently.

Activity Measurement:

1) The tubes are thawed in an ice bath and centrifuged at full speed for 5 min. Samples should be maintained at approximately 0°C during analysis of enzyme activity.

2) 2.5 mL of the supernatant is decanted into a cuvette and 0.25 mL of NaOH/Borax buffer is added.

3) Fluorescence is measured at 455 nm emission with 365 nm excitation. A standard curve is prepared using known dilutions of the 1 uM standard and the fluorescence intensity plotted against MUF concentration. The MUF concentration in the samples is calculated by comparing the fluorescence to the standard curve.

4) To determine dry weight of the sediment, the preweighed tubes with sediment are placed in a convection oven and dried overnight to constant weight.

Results Calculation:

1) Exoenzyme activity is calculated as uM product/g sediment/h + /- S.D.

2) Exoenzyme activity is plotted against metal concentration and the EC_{50} value calculated from a regression of % depression vs log metal concentration.

APPENDIX B: Method for Exoenzyme Activity Bioassay in Sediment Cores

Introduction: While the majority of methods for toxicity assessment in sediments employ suspended sediments (slurries) this is not representative of the natural conditions in marine sediments. Intact sediments are characterized by a highly structured environment that favours the establishment of chemical microgradients in the interstitial water and the resultant microclimates greatly affect the metabolism and ecology of the sediment microbiota. These microgradients and climates are destroyed when sediments are suspended in slurries and the measured activities in slurries and cores are markedly different.

The basis of this bioassay is similar to that described in Appendix A for use in sediment slurries, but has the advantage that measurement is accomplished within intact cores of sediment thus providing enzyme activity and toxicity estimates more representative of natural conditions.

Outline of Method: Known dilutions of a toxicant are injected into the center of sediment cores at 0.5 cm intervals. After a preincubation period, a 5 mM methylumbelliferyl (MUF) substrate is injected, at identical intervals, into the core. Following incubation, the core is frozen and cut into 1cm sections. A 5.0 mm plug of frozen sediment is removed from the centre of each segment using a cork drill and suspended in synthetic sea water. Following centrifugation the concentration of MUF in the supernatant was measured by fluorescence spectroscopy (Fig. 52). Enzyme activity is calculated via quantification of the amount of liberated MUF. Exoenzyme activity profiles are then plotted and the EC_{50}, the toxicant concentration resulting in a 50% enzyme activity decrease, is calculated for each sediment horizon.

Reagents:

Synthetic Ocean Water (SOW) is prepared according to Morel et al. (1979). Reagent grade salts are added, in the following order, to 1L of distilled water:

Salt	Amount (g)
NaCl	24.53
$CaCl_2.2H_2O$	1.54
KBr	0.10
NaF	0.003
KCl	0.70
H_3BO_3	0.03
Na_2SO_4	4.09
$NaHCO_3$	0.20
$SrCl_2.6H_2O$	0.017
$MgCl_2.6H_2O$	11.10

The mixture is adjusted to pH 8.0 using 1N $NaHCO_3$.

Metal stock solutions used as reference toxicants in the present study are prepared in SOW as follows:

Salt	Stock Concentration (M)
$HgCl_2$	0.0015
$CuCl_2.2H_2O$	0.0047
$CdSO_4$	0.0267
$ZnSO_4.7H_2O$	0.0459
$PbCl_2$*	0.0145
$NiCL_2.6H_2O$	0.0511

* Prepared in distilled water to prevent precipitation.

Methylumbelliferyl substrate stocks are prepared using 4-Methylumbelliferyl-B-D-glucoside, 4-Methylumbelliferyl-phosphate (Sigma Chemical Co.) and L-Leucine-4-methyl coumarinyl-7-amide HCl (Fluka Chemical Corp.), at a concentration of 5mM in 10% methanol in SOW. MUF substrates are stored at -20°C when not in use.

NaOH/Borax Buffer (pH 10.8) is prepared with 0.2M NaOH and 0.05M sodium tetraborate. The buffer consists of 50mL of sodium tetraborate solution and 67mL of NaOH and adjusted to a final volume of 200mL with distilled water.

Methylumbelliferone Standard solution (2.5mM) was prepared using methylumbelliferone (Sigma Chemical Co.) in reagent grade methanol. A working solution was prepared by diluting 200uL of the stock in 200mL of SOW. The final standard solution was prepared with 1.2 mL of working solution and 1.8 mL SOW. This yielded the required 3.0 mL assay volume at a concentration of 1 uM.

All glassware used in the study is soaked overnight in 5% HNO_3 and rinsed at least 5 times with distilled water.

Sediment Cores: Sediment core tubes are constructed from 12.5mm (I.D.) polycarbonate tubing. At 5mm intervals, sampling ports are made by drilling 0.5mm holes which were filled with silicone sealant (General Electric Co.). After sampling, the bottom of the core tubes is plugged with rubber stoppers.

Sediment Preparation: At least 8 hours prior to analysis, experimental cores are subsampled from large cores (9.0 cm dia.) collected in the field. Cores of approximately 8cm depth are taken and stored at ambient temperature until used for analysis.

All sediment cores are stored submerged in running sea water tanks until used.

Assay Procedure:

1) Known dilutions of toxicant stocks are made to yield a range of appropriate concentrations, once injected into the sediment cores, as follows,

Dilution	Final Concentration (ppm)*
1:100	1.0
1:20	5.0
1:7	12.5
1:1	50.0
0	100.0

* These concentrations are based on an interstitial water volume of 0.5 mL in each horizon. The actual water volume must be measured and the correct toxicant concentration calculated. The given concentrations are for Zn, Cd, Pb, or Ni. The resultant concentrations of Cu and Hg are one tenth of those shown above.

2) At 0.5 cm intervals, 10 uL aliquots of each dilution of toxicant are injected into triplicate cores using a repeating pipetter fitted with a syringe needle. The cores are incubated at ambient temperature for 30 min.

3) After preincubation, 10 ul aliquots of 5 mM MUF-substrates are injected at each port and the time recorded prior to reincubation.

4) After 60 min the cores are frozen in dry ice to arrest enzymatic activity. Once frozen the cores may be stored below 0°C until activity measurement by fluorescence spectroscopy.

Activity Measurement:

1) Samples must be maintained at aproximately 0°C during measurement of activity. The frozen cores are removed from the core tubes and sectioned into 1 cm horizons. A 5.0 mm plug is removed from the centre of each horizon with a cork drill and suspended in 3.0 mL of SOW in preweighed test tubes. The tubes are maintained in an ice bath until centrifuged (4750 rpm; 5 min.).

2) Experimental blanks (SOW with sediment) and a 1.0 uM MUF standard are prepared concurrently.

3) 2.5 mL of the supernatant is decanted into a cuvette and 0.25 mL of NaOH/Borax buffer is added.

4) Fluorescence is measured at 455 nm emission with 365 nm excitation. A standard curve is prepared using known dilutions of the 1 uM standard and the fluorescence intensity plotted against MUF concentration. The MUF concentration in the samples is calculated by comparing the fluorescence to the standard curve.

5) The preweighed tubes with sediment are placed in a convection oven and dried overnight to constant weight to determine dry weight.

Results Calculation:

1) Exoenzyme activity is calculated as uM product/g sediment/h + /- S.D. and is plotted against sediment depth to form activitiy-depth profiles.

2) Exoenzyme activity in a given sediment horizon is plotted against metal concentration and the EC_{50} value calculated from a regression of % depression vs log metal concentration.

APPENDIX C: Method for Radiolabelled Organic Uptake Bioassay in Sediments.

Introduction: The uptake of radiolabelled organic molecules, such as ^{14}C-glucose, ^{14}C-glutamic acid and ^{3}H-thymidine, has been used extensively for the assessment of microbial activity in aquatic environments.

In the following method, the effects of toxicants on sediment bacterial communities are assessed through quantification of radiolabelled thymidine and glutamic acid incorporation. The high specific activity of these compounds allows measurement of uptake rates at low substrate concentrations similar to those found in nature.

Outline of Method: A toxicant is added to samples of sediment suspended in synthetic sea water and incubated in the presence of 0.022 uCi/mL of ^{14}C-glutamic acid or 0.37 uCi/mL of ^{3}H-thymidine. For analysis of ^{14}C-glutamic acid uptake, the samples are acidified following incubation to arrest metabolic activity and drive off respired CO_2. Total uptake (cellular uptake and respiration) of glutamic acid is quantified by measuring the radioactivity activity of filtered samples and recovered CO_2. Similarily, heterotrophic activity was also estimated by measuring the incorportion rates of ^{3}H-thymidine incorporation into DNA (Fig. 53). Compound toxicity is calculated as the EC_{50}, the toxicant concentration resulting in a 50% decrease in microbial activity.

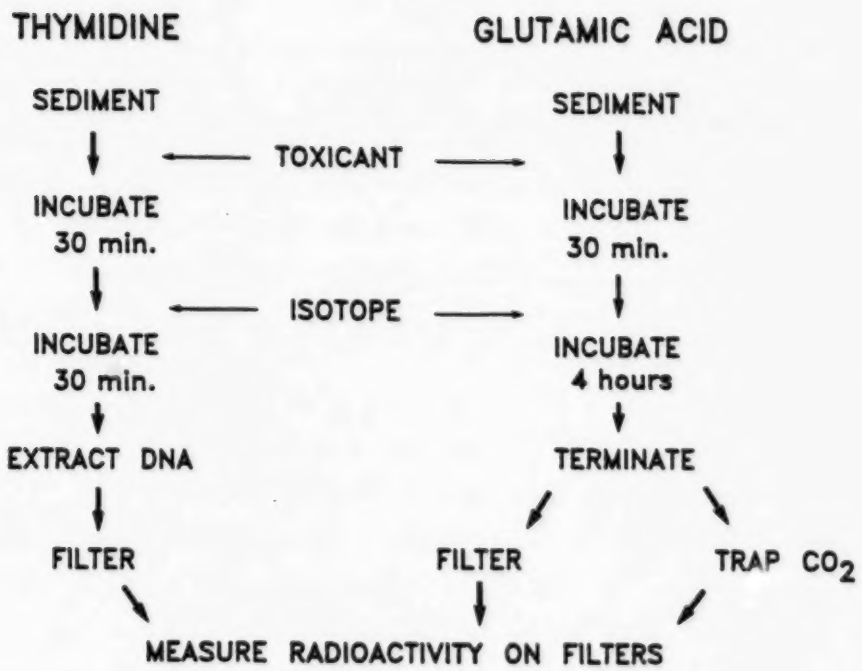

Figure 53: Outline of Radiolabelled Organic Uptake Bioassay Protocol.

Reagents

Synthetic Ocean Water (SOW) is prepared according to Morel et al. (1979). Reagent grade salts are added, in the following order, to 1 L of distilled water.

Salt	Amount (g)
NaCl	24.53
$CaCl_2.2H_2O$	1.54
KBr	0.10
NaF	0.003
KCl	0.70
H_3BO_3	0.03
Na_2SO_4	4.09
$NaHCO_3$	0.20
$SrCl_2.6H_2O$	0.017
$MgCl_2.6H_2O$	11.10

The mixture is adjusted to pH 8.0 using 1N $NaHCO_3$.

Metal stock solutions used as reference toxicants in the present study are prepared in SOW as follows:

Salt	Stock Concentration (M)
$HgCl_2$	0.0015
$CuCl_2.2H_2O$	0.0047
$CdSO_4$	0.0267
$ZnSO_4.7H_2O$	0.0459
$PbCl_2$*	0.0145
$NiCL_2.6H_2O$	0.0511

* Prepared in distilled water to prevent precipitation.

Radiolabelled Organic Substrates were prepared from high specific activity stocks of ^{14}C-Glutamic Acid (>290 mCi/mmol) and methyl-^{3}H-thymidine (>80 Ci/mmol) in 0.22um filtered distilled water. Working solutions were prepared on the day of use as follows,

	Concentration (uM)	Activity (uCi/mL)
^{14}C-Glutamic Acid	34	>9.9
^{3}H-Thymidine	1.6	>128

Trichloroacetic Acid (TCA) is prepared in a 10% (w/v) solution using trichloroacetic acid (BDH Chemicals Ltd.) in distilled water. This solution is stored on ice until used.

Sulfuric Acid is prepared as a 2 N solution in distilled water.

Sediment Preparation: Fresh sediment cores are stored in running sea water tanks until required for experimental analysis, at which time a 0-2 cm layer of the sediment cores is removed, homogeneously mixed in a beaker with a glass stirring rod, and maintained at ambient temperatures.

Assay Procedure:

1) Experimental samples are prepared in triplicate (in 25 ml test tubes) as follows for all metals except lead:

Metal Sol'n (uL)	SOW (mL)
0	9.0
30	8.97
150	8.85
300	8.7
1500	7.5
3000	6.0

Since the lead stock solution is prepared in distilled water, it is necessary to ensure that the salinity in each tube is the same. To accomplish this, 6.0mL of SOW is added to each tube, the appropriate amount of lead stock is then added and the volume brought to 9.0mL with distilled water. When dispensed as above, these stock solutions yield concentrations of 1, 5, 10, 50 and 100 ppm of Hg and Cu and 10, 50, 100, 500, and 1000 ppm of Cd, Zn, Pb and Ni. Duplicate sets are prepared for thymidine and glutamic acid.

2) Thoroughly mixed sediment (1.25 g wet weight) is added to each tube and the tubes are fitted with rubber septa. The tubes for glutamic acid uptake are also fitted with a nylon center well (Kontes Scientific Glassware Ltd.) containing an accordian folded 25 mm GF/C filter (Whatman International Co.).

3) Each tube is agitated for about 5 sec using a vortex mixer and incubated for 30 min at ambient temperature.

Glutamic acid Uptake After the 30 min preincubation, 20 uL of working isotope solution is added to each tube and the tubes are agitated and incubated at ambient temperature for 4 h. Blank tubes are prepared as above except 1 mL of 2 N H_2SO_4 is added immediately prior to isotope addition.

After the incubation period, 1 mL of 2 N H_2SO_4 is injected through the septum into each tube to arrest microbial activity and liberate respired CO_2. Phenethylamine (0.1mL;

Terochem Laboatories Ltd.) is injected into the centre well to absorb liberated CO_2.

After 12 h, filters are removed from the centre wells and placed in scintillation vials with 8 mL of Ready-Protein + (Beckman Instruments Inc.) scintillation fluor. Sediment is filtered onto 0.22 um cellulose nitrate filters (Millipore Corp.) rinsed with 0.22 um filtered SOW and treated as above. Activity is measured in a Beckman LS-3801 scintillation counter.

Thymidine Incorporation After the 30 min pre incubation, 25 uL of working isotope solution is added to each tube and incubated for 30 min. Blanks are prepared by adding 70 uL of formaldehyde before isotope addition.

After incubation, 10 mL of cold TCA is added to each tube to precipitate DNA and arrest biological activity. Tubes are incubated in an ice bath for 20 min, filtered onto GF/C filters and rinsed with 5 ml of cold 5% TCA. Radioactivity on the filters was then measured by liquid scintillation counting as described above.

Results Calculation:

1) Glutamic acid uptake is calculated as the sum of incorporated ^{14}C (on filters) and the respired $^{14}CO_2$ (centre well filter) and expressed as DPM/g sediment/h +/- S.D.

2) Thymidine uptake is calculated as the amount incorporated into the cold TCA precipitable fraction (DNA) and expressed as DPM/g sediment/h +/- S.D.

3) Radioisotope uptake is plotted against toxicant concentration and the EC_{50} value calculated from a regression of % depression vs log metal concentration.

APPENDIX D: BASIC Microcomputer Program for Calculating EC_{50} Values from Bioassay Data.

The following program is written in Microsoft(R) GWBASIC for the IBM PC(R) Microcomputer. This interactive program accepts the data resulting from the exoenzyme and radiolabelled organic bioassays and calculates the mean activity +/- S.D., the regression of toxicant concentration vs mean activity and the EC_{50} value. Sample output is provided following the program listing.

```
5 REM TOXICITY CALCULATION PROGRAM EC50.BAS WRITTEN IN GWBASIC
6 REM
10 CLEAR : CLS
20 REM
55 DIM TUBE (30), MUF(4,30), CONC$(10), RESULTS(6,10), HOLD(10), GLAF(30), SED(3
0), DPM(10), DURATION(30)
60 GOSUB 8000
70 CLS : INPUT "Date of Experiment: "; ATE$
80 IF RESP$ = "I" THEN PRINT : INPUT "Incubation Time (min) "; DUR
85 PRINT : INPUT "Number of replicates used at each conc. ";NREP
90 PRINT : INPUT "Sediment used ";SEDIMENT$
100 PRINT : INPUT "Substrate used (eg. MUF-glu) ";SUBST$
110 PRINT : INPUT "Incubator Temperature ";HEAT
125 PRINT
130 PRINT"Toxicant used and # of concentrations used"
135 INPUT "excluding control "; TOX$, NCONC : CONC$(1) = "CTRL"
140 PRINT : PRINT"Enter concentrations used (in ppm) "
145 PRINT : FOR A = 2 TO (NCONC +1) : INPUT CONC$(A) : NEXT A
150 CLS : REM ENTER MUF VALUES
160 NT = (NCONC + 1) * NREP
165 IF RESP$ = "I" THEN GOSUB 7000 : GOTO 360
200 PRINT"Now enter the concentration of MUF" : PRINT"(in uMol) for each tube" :
 PRINT : PRINT
210 FOR V = 1 TO NT
220 PRINT "Tube ";V; : INPUT  ATA$
225 IF ATA$ = "LOST" THEN GLAF(V) = 1
226 IF GLAF(V) = 1 THEN MUF(1,V) = 0 ELSE  MUF(1,V) = VAL(ATA$)
230 PRINT : MUF(2,V) = MUF(1,V) * 3
235 NEXT V : PRINT : PRINT : PRINT
240 BBB = NT/2 : BBB% = BBB : RES = BBB - BBB% : IF RES = 0 THEN 250 ELSE BBB =
(NT/2)-.5
250 FOR C = 1 TO (BBB) : PRINT "TUBE "; C;"  ";     MUF(1,C) ,"TUBE ";(C+BBB); "
  ";MUF(1,(C+BBB)) : NEXT C : IF RES < 0 THEN PRINT , , "TUBE "; NT; "  "; MUF(1
,NT)
260 PRINT     : INPUT "Are these values correct (Y/N) ?"; ANS$
270 IF ANS$ = "Y" THEN GOTO 300 ELSE PRINT : INPUT "WHICH VALUE IS INCORRECT? ";
NUM
```

```
280 PRINT : PRINT "NEW VALUE FOR TUBE ";NUM; : INPUT CORR
290 MUF(1,NUM) = CORR : MUF (2,NUM) = CORR * 3 : PRINT : PRINT : GOTO 250
295 GOSUB 6000
300 CLS : PRINT"Now enter the dry weights of the tubes with sediment"
310 IF SER$ = "A" THEN SER = 1 ELSE IF SER$ = "B" THEN SER = 2 ELSE IF SER$ = "C
" THEN SER = 3 ELSE IF SER$ = "D" THEN SER = 4 ELSE IF SER$ = "F" THEN SER = 5 E
LSE SER = 6
320 PRINT : PRINT : FOR E = 1 TO NT : PRINT : PRINT"TUBE ";E ; : INPUT SED(E) :
NEXT E
321 PRINT : PRINT : PRINT : FOR W = 1 TO BBB : PRINT"TUBE "; W ; "  "; SED(W),"T
UBE "; (W+ BBB); "  "; SED(W+BBB) : NEXT W : IF RES < 0 THEN PRINT , , "TUBE ";N
T; "  "; SED(NT)
325 PRINT : PRINT : INPUT "Are all these values correct "; ANS$ : IF ANS$ = "Y"
THEN 328 ELSE INPUT "Which one is incorrect "; NUM : INPUT "Enter correct value
";SED(NUM) : GOTO 321
328 GOSUB 3100
330 FOR E = 1 TO NT : MUF(3,E) = SED(E) - TUBE(E)
340 MUF(4,E) = (MUF(2,E)/MUF(3,E)) * (60/DURATION(E))
350 NEXT E
360 REM CALCULATE RESULTS
400 CLS : FOR Q = 1 TO 10 : PRINT : NEXT Q : PRINT"        Hang on while I figure
 this out. . . ."
490 F = 0
500 FOR X = 1 TO (NCONC + 1)
510 MEAN = 0
520 ZEE = NREP
530 FOR G = 1 TO NREP
540 F = F + 1
550 HOLD(G) = MUF (4,F)
555 IF GLAF(F) = 1 THEN ZEE = ZEE - 1
560 MEAN = MEAN + HOLD(G) : NEXT G
570 IF X = 1 THEN RESULTS (4,X) = 0 : GOTO 600
580 RESULTS(4,X) = LOG(VAL(CONC$(X)))/2.303
600 RESULTS(1,X) = MEAN/ZEE
620 RESULTS(2,X) = (RESULTS(1,1) - RESULTS(1,X)) * 100/ RESULTS(1,1) * -1
625 S = 0
630 FOR H = 1 TO NREP : HOLD(H) = (HOLD(H) - RESULTS(1,X))*(HOLD(H) - RESULTS(1,
X)) : S = S + HOLD(H) : HOLD(H) = 0 : NEXT H
635 S = SQR(S/( ZEE-1)) : RESULTS(3,X) = S
640 IF F = NT THEN 1000
660 NEXT X
670 REM END RESULTS CALCULATION
680 REM
690 REM
1000 REM LINEAR REGRESSION
1001 REM
1010 EX = 0 : EY = 0 : EXY = 0 : EX2 = 0 : M = 0 : B = 0 : R = 0 : EY2 = 0
1020 FOR I = 1 TO NCONC + 1
1030 EX = EX + RESULTS (4,I) : EY = EY + RESULTS (2,I) : EXY = EXY + (RESULTS(4,
I) * RESULTS(2,I)) : EX2 = EX2 + (RESULTS(4,I)*RESULTS(4,I)) : EY2 = EY2 + (RESU
LTS(2,I) * RESULTS (2,I)) : NEXT I
```

```
1040 M = (NCONC * EXY - (EX * EY)) / (NCONC * EX2 - (EX*EX))
1045 M% = M : DIF = M - M% : DIF% = DIF * 1000 : DIF = DIF% / 1000 : M = M + DIF
1050 B = (EY/NCONC) - (M * (EX/NCONC))
1055 B% = B : DIF = B - B% : DIF% = DIF * 1000 : DIF = DIF% / 1000 : B = B% + DI
F
1060 R =((NCONC * EXY) - (EX * EY))/ SQR((NCONC * EX2 - (EX*EX)) * (NCONC * EY2
- (EY*EY)))
1065 R% = R * 1000 : R = R% / 1000
1070 EC50 = (-50 - B) / M : EC50 = EC50 * 2.303 : EESEE = EXP(EC50)
1075 EC50 = EESEE
1090 REM
1100 REM
2000 REM OUTPUT OF RESULTS TO DISK FILE
2001 REM
2010 OPEN "O", #1,"TOX.DAT"
2015 PRINT #1, NT, (NCONC + 1)
2018 GOSUB 3500
2020 FOR L = 1 TO NT : PRINT #1, MUF(1,L), MUF(3,L), MUF(4,L) : NEXT L
2040 FOR W = 1 TO NCONC + 1 : PRINT #1, CONC$(W) : PRINT #1,   RESULTS(1,W),  RE
SULTS(3,W), RESULTS(2,W) : NEXT W
2050 PRINT #1, M, B, R, EC50
2060 CLOSE #1
2070 REM END DATA OUTPUT TO DISK
2080 REM
2090 CLS : INPUT "What do you wisk to call data file ?"; NOM$
2095 NAME "TOX.DAT" AS NOM$
2100 GOSUB 8000
3000 END
3010 REM
3020 REM
3100 CLS : PRINT "Now input the incubation time for each tube ": PRINT : PRINT :
 FOR X = 1 TO NT : PRINT : PRINT "TUBE "; X; "   "; : INPUT DURATION(X) : NEXT X
: CLS
3110 FOR C = 1 TO (BBB) : PRINT"TUBE "; C; "    "; DURATION(C), "TUBE "; C+BBB; "
   "; DURATION(C+BBB) : NEXT C : IF RES < 0 THEN PRINT , , "TUBE "; NT; "    "; D
URATION (NT)
3120 PRINT : INPUT " ARE THESE VALUES CORRECT  (Y/N) "; ANS$ : IF ANS$ = "Y" THE
N GOTO 3130 : ELSE PRINT : INPUT "WHICH VALUE IS INCORRECT "; NUM : PRINT : PRIN
T "NEW VALUE FOR TUBE ";NUM; : INPUT DURATION(NUM) : GOTO 3110
3130 REM
3140 CLS : PRINT "NOW ENTER THE WEIGHTS OF THE EMPTY TUBES "
3150 PRINT : PRINT : FOR X = 1 TO NT : PRINT : PRINT"TUBE "; X; "   "; : INPUT TU
BE (X) : NEXT X : PRINT : PRINT : PRINT
3160 FOR C = 1 TO BBB : PRINT "TUBE "; C; "   " ; TUBE(C), "TUBE "; C+BBB; "   ";
TUBE(C+BBB) : NEXT C : IF RES < 0 THEN PRINT , , "TUBE "; NY; "   "; TUBE(NT)
3170 PRINT : INPUT "ARE THESE VALUES CORRECT  (Y/N) "; ANS$ : IF ANS$ = "Y" THEN
 3190 ELSE PRINT : INPUT "WHICH ONE IS INCORRECT ";NUM : PRINT : PRINT"CORRECT V
ALUE FOR TUBE "; NUM; : INPUT TUBE (NUM) : GOTO 3160
3190 RETURN
3500 PRINT #1, SUBST$ : PRINT #1, ATE$ : PRINT #1, SEDIMENT$ : PRINT #1, HEAT :
PRINT #1, TOX$ : IF RESP$ = "A" THEN PRINT #1, RESP$ ELSE PRINT #1, ISOT$
3510 RETURN
```

```
5000 REM PRINTER OUTPUT ROUTINE
5001 REM
5010 CLS : INPUT "NAME OF DATA FILE"; FILE$
5020 OPEN "I", #1, FILE$
5030 INPUT #1, NT, NCONC
5040 LPRINT,"TOXICITY DATA FILE ";FILE$ : LPRINT : LPRINT
5045 GOSUB 5400 : IF RESP$ = "T" THEN LPRINT "                                                    DPM
      SED. WEIGHT (G)     UPTAKE (DPM/G/H)" : GOTO 5060
5046 IF RESP$ = "A" THEN LPRINT "                                  MUF CONC. (uM)      SED
. WEIGHT (g)   ACTIVITY (nM/g/h)" ELSE LPRINT "                                    UPTAKE (D
PM/g/h)      RESPIRATION       GROSS ACTIVITY"
5060 FOR P = 1 TO NT : LPRINT : LPRINT,"Tube "; P; "        "; : FOR Q = 1 TO 3 :
 INPUT #1, TEMP : LPRINT TEMP; "          "; : NEXT Q : LPRINT " " : NEXT P
5070 LPRINT CHR$(12) : FOR U = 1 TO 5 : LPRINT : NEXT U
5080  LPRINT,"TOXICITY RESULTS OF "; FILE$ : LPRINT : LPRINT
5090 LPRINT,"    [TOXICANT]      MEAN ACT.          STD. DEV.           % EFFECT"
5100 FOR S = 1 TO NCONC : LPRINT : INPUT #1, CON$ : LPRINT,"       "; CON$; "
     "; :FOR T = 1 TO 3 : INPUT #1, TEMP : LPRINT TEMP; "         "; : NEXT T
5105 LPRINT " " : NEXT S
5110 FOR U = 1 TO 5 : LPRINT : NEXT U
5120 LPRINT,"REGRESSION OF % EFFECT vs log [Toxicant]"
5130 LPRINT : INPUT #1, M, B, R, EC50 : LPRINT : LPRINT,"F(x) = "; M; "X";
5140 IF B<0 THEN LPRINT " "; : ELSE LPRINT " + ";
5150 LPRINT B : LPRINT : LPRINT,"r = "; R
5160 LPRINT : LPRINT,"EC50 = "; EC50; "   mg/L"
5165 LPRINT CHR$(12)
5170 CLOSE #1
5175 RESP$ = ""
5180 RETURN
5400 LPRINT : INPUT #1, SUBST$, ATE$, SEDIMENT$, HEAT, TOX$, RESP$
5410 LPRINT,"Substrate:"; SUBST$; "                    "; ATE$
5420 LPRINT,"Sediment:"; SEDIMENT$
5430 LPRINT,"Incubator Temperature "; HEAT; "C                    Toxicant: "; TOX$
5440 LPRINT
5450 RETURN
6000 REM GET TUBE WIGHTS
6010 CLS : PRINT TAB(25) "Now enter weights of empty tubes "
6020 FOR D = 1 TO NT : PRINT "TUBE "; D; : INPUT TUBE (D) : NEXT D
6030 FOR V = 1 TO BBB : PRINT"TUBE ";V; "   "; TUBE(V), "TUBE ";V; "   "; TUBE(V+B
BB) : NEXT V
6040 IF RES < 0 THEN PRINT, , "TUBE ";NT; "   "; TUBE(NT)
6050 PRINT : PRINT : INPUT ARE ALL THESE VALUES CORRECT "; ANS$ : IF ANS$ = "Y"
THEN 6060 ELSE INPUT WHICH ONE IS INCORRECT "; NUM : INPUT "ENTER CORRECT VALUE
"; TUBE(NUM) : GOTO 6030
6060 RETURN
7000 REM ISOTOPE INPUT
7005 CLS : PRINT : PRINT"          ISOTOPE DATA INPUT" : PRINT : PRINT
7010 PRINT : INPUT "DRY WEIGHT OF SEDIMENT USED ";WEIGHT
7020 PRINT : INPUT "Is data for T)hymidine, G)lutamic acid or gl(U)cose  (T,G,U)
 "; ISOT$
7030 IF ISOT$ = "T" THEN 7035 ELSE 7100
7035 L = 0
```

```
7040 FOR N = 1 TO NCONC + 1 : PRINT : PRINT" TOXICANT CONC. #"; N; " UPTAKE" : F
OR M = 1 TO NREP : INPUT "  DPM "; DPM(M) : NEXT M : INPUT "  BKG "; BKG
7045 LLL = 1 : NNN = NREP : PRINT : GOSUB 7800
7050 FOR M = 1 TO NREP : L = L + 1 : MUF(1,L) = DPM(M) : MUF(2,L) = DPM(M) - BKG
 : MUF(3,L) = WEIGHT : MUF(4,L) = MUF(2,L)/MUF(3,L) * (60/DUR) : NEXT M : NEXT N
7060 GOTO 7500
7100 Z = 0
7110 FOR X = 1 TO NCONC + 1 : PRINT : PRINT"TOXICANT CONC. #"; X; " UPTAKE" : FO
R Y = 1 TO NREP : INPUT "  DPM "; DPM(Y) : NEXT Y : INPUT "  BKG "; BKG : PRINT
7112 LLL = 1 : NNN = NREP : GOSUB 7800 : PRINT : PRINT
7114 PRINT "TOXICANT CONC. #";X; " RESPIRATION"
7115 FOR A = 1 TO NREP : DPM(A) = DPM(A) - BKG : INPUT "  DPM "; DPM(A+NREP) : N
EXT A : INPUT "  BKG "; BKG : LLL = NREP + 1 : NNN = 2 * NREP : PRINT : PRINT :
GOSUB 7800
7118 FOR A = 1 TO NREP : DPM (A+NREP) = DPM(A+NREP) - BKG : NEXT A
7120 FOR Y = 1 TO NREP : Z= Z+ 1 : MUF(1,Z) = DPM(Y)/WEIGHT * 60/ DUR : MUF (3,Z
) = DPM(Y + NREP) / WEIGHT * 60 / DUR : MUF (4,Z) = MUF(1,Z) + MUF(3,Z) : NEXT Y
 : NEXT X
7500 RETURN
7800 FOR U = LLL TO NNN : PRINT U; "  DPM "; DPM(U) : NEXT U : PRINT NNN+1; "  B
KG "; : PRINT BKG : INPUT "ARE ALL THESE VALUES CORRECT "; ANS$ : IF ANS$ = "Y"
THEN 7820 ELSE INPUT "WHICH VALUE IS INCORRECT ";NUM : INPUT "CORRECT VALUE ";AA
A
7810 IF NUM < (NNN+1) THEN DPM(NUM) = AAA ELSE BKG = AAA
7815 GOTO 7800
7820 CLS
7830 RETURN
8000 REM STARTING MENU
8010 CLS : RESP$ = ""
8020 PRINT "#"; : FOR N = 1 TO 50 : PRINT"#";: NEXT N : PRINT"#"
8025 PRINT "#          MICROBIAL ACTIVITY BIOASSAY              #" : PRINT "#"; :
 FOR N = 1 TO 50 : PRINT "#"; : NEXT N : PRINT "#"
8030 FOR N = 1 TO 2 : PRINT"#                                                    #
" : NEXT N : PRINT"#           A - Analyse Enzyme Data                #" : PRINT
   "#                                                  #"
8035 PRINT"#           1 - Analyse Isotope Data                #" : PRINT"#
                                                  #"
8040 PRINT"#           S - Output Data to Screen               #" : PRINT"#
                                                  #"
8045 PRINT"#           P - Output Data to Printer              #" : PRINT"#
                                                  #"
8050 PRINT"#           Q - Quit                                #" : FOR N = 1 TO
3 : PRINT"#                                                   #" : NEXT N
8055 PRINT "#"; : FOR N = 1 TO 50 : PRINT"#"; : NEXT N : PRINT"#"
8060 INPUT RESP$ : IF RESP$ = "Q" THEN GOTO 3000
8061 IF RESP$ ="P" THEN GOSUB 5000
8062 IF RESP$ = "S" THEN GOSUB 9000
8063 IF RESP$ = "A" THEN GOTO 70
8065 IF RESP$ = "I" THEN GOTO 70 ELSE GOTO 8010
8070 RETURN
9000 REM SCREEN OUTPUT
9010 CLS : INPUT "Name of Data File"; FILE$
9035 OPEN "I", #1, FILE$
9040 INPUT #1, NT, NCONC
```

```
9050 GOSUB 9200
9060 FOR P = 1 TO NT : PRINT P; : FOR Q = 1 TO 3 :  INPUT #1, TEMP : PRINT"
    "; TEMP; : NEXT Q : PRINT" " : FLAG = FLAG + 1
9070 IF FLAG = 10 THEN GOSUB 9500
9075 NEXT P : GOSUB 9500
9080 CLS
9090 PRINT"   TOXICITY RESULTS OF "; FILE$
9100 PRINT : PRINT"[TOXICANT]","MEAN ACT.","STD. DEV.","% EFFECT"
9110 FOR S = 1 TO NCONC : PRINT : INPUT #1, CON$ : PRINT CON$;"          ";:FOR T
 = 1 TO 3 : INPUT #1, TEMP : PRINT  TEMP; "          "; : NEXT T : PRINT " "
9111 NEXT S
9120 PRINT : PRINT"REGRESSION OF % EFFECT VS LOG[TOXICANT]"  : PRINT : INPUT #1,
 M, B, R, EC50
9130 PRINT"F(x) = "; M; "x "; : IF B > 0 THEN PRINT"+ "; : PRINT B : PRINT : PRI
NT"r = "; R ELSE PRINT B : PRINT : PRINT "r = "; R
9140 PRINT : PRINT"EC50 = "; EC50; "  mg/L"
9150 CLOSE #1
9155 GOSUB 9500
9160 RETURN
9170 REM
9180 REM
9190 REM
9200 INPUT #1, SUBST$, ATE$, SEDIMENT$, HEAT, TOX$, RESP$
9210 PRINT"TOXICITY DATA FILE "; FILE$
9220 PRINT : IF RESP$ = "T" THEN PRINT"TUBE ", "DPM", "SED. WGT (g)", "ACTIVITY
(DPM/g/h)" :GOTO 9240
9230 IF RESP$ = "A"         THEN PRINT"TUBE ","MUF (uM)","SED. WGT (g)","ACTIVITY
(nM/g/h)" ELSE PRINT "TUBE ", "UPTAKE " ,"RESP. ","GROSS ACTIVITY (DPM/g/h)"
9240 FLAG = 0
9250 RETURN
9490 REM
9495 REM
9500 REM WAIT ROUTINE
9510 PRINT"  PRESS SPACE BAR TO CONTINUE " : C$ = INPUT$(1)
9515 RESP$ = ""
9520 RETURN
9530 REM
9540 REM
```

Sample output from EC50.BAS program

TOXICITY DATA FILE LEU.HG

Substrate: MCA-LEU 3-12-87
Sediment: LONG COVE CORE I (sandy)
Incubator Temperature 7 C Toxicant: MERCURY

	MUF CONC. (uM)	SED. WEIGHT (g)	ACTIVITY (nM/g/h)
Tube 1	.37	.363	2.293
Tube 2	.4	.342	2.632
Tube 3	.42	.351	2.692
Tube 4	.24	.355	1.521
Tube 5	.25	.377	1.492
Tube 6	.25	.301	1.869
Tube 7	.19	.3	1.425
Tube 8	.2	.301	1.495
Tube 9	.18	.272	1.489
Tube 10	.14	.214	1.472
Tube 11	.15	.269	1.255
Tube 12	.17	.281	1.361
Tube 13	.15	.31	1.089
Tube 14	.13	.252	1.161
Tube 15	.16	.328	1.079
Tube 16	.14	.292	1.079
Tube 17	.16	.348	1.034
Tube 18	.13	.292	1.002

TOXICITY RESULTS OF LEU.HG

[TOXICANT]	MEAN ACT.	STD. DEV.	% EFFECT
CTRL	2.539	.215	0
1	1.627	.21	-35.91
5	1.47	.039	-42.119
10	1.363	.109	-46.335
50	1.116	.039	-56.061
100	1.038	.039	-59.107

REGRESSION OF % EFFECT vs log [Toxicant]

F(x) = -12.058 x -34.891

r = -0.996

EC50 = 17.9 mg/L